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SUMMATION AND THE ABSENCE OF A REFRACTORY PERIOD IN ISOLATED SKELETAL MUSCLE FIBERS [1, 2]

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The isometric twitch-like response of an isolated length of a single muscle fiber with cut ends has been shown to resemble in its form and in its time relations the isometric twitch of the whole muscle (Brown and Sichel, 1930, 1936; Gelfan, 1933). This has led to the view that the release of energy involves the same ultimate mechanism in both cases. The twitch of the isolated length of fiber differs from that of the intact muscle primarily in that in the isolated fiber the twitch may be graded and not be propagated (Gelfan, 1930; Brown and Sichel, 1936; Sichel and Prosser, 1935). In each excited fiber of the intact muscle, however, stimulation starts a propagated wave of excitation which results in an all-or-nothing response and a refractory period, and is associated with a propagated action potential. It is important to consider further the differences between the excitation of the non-conducted response of the isolated fiber and the normal conducted response of the whole muscle. We have, therefore, examined the isolated fiber to ascertain whether its excitation is or is not followed by a refractory period. The results indicate that there is no refractory period associated with the graded, non-propagated response. These differences in behavior from that of the whole muscle suggest the advisability of studying summation in the single fiber. The absence of a conducted response and of a refractory period makes possible types of summation which cannot occur in the intact muscle. The local nature of the response makes possible a

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spatial summation; the absence of any refractory period allows temporal summation in an interval during which, in whole muscle, a second stimulus could contribute nothing to the response. Since we are dealing here with direct excitation of the contractile system without involving the conduction mechanism, these properties of summation are important in an analysis of the contractile mechanism.

PREPARATION AND METHODS. The material chosen for the investigation of this problem was a length of a single fiber isolated from the adductor longus of the bullfrog (R. catesbiana). The fiber was immersed in a mixture of equal parts of turtle serum and Ringer's solution and mounted with but minor modifications in the manner previously described (Brown and Sichel, 1930, 1936; Sichel, 1934). To afford control of the temperature, the chamber in which the fiber was mounted was jacketed so that it could be cooled by circulating brine.

The fiber was placed under a slight initial tension and was stimulated with silver electrodes.[3] These were sheets about 0.1 mm. in thickness, the surfaces of which were insulated except for one edge on each electrode. The electrodes were placed so the fiber lay between them and in their plane, the uninsulated edges being 0.05 mm. to 0.10 mm. away from the fiber, and parallel to its longitudinal axis, so that the direction of the field was at right angles to this axis. The contractions were recorded in a manner similar to that previously reported, the movement of the lever being magnified 900 times on the record. The recording was isometric, the greatest tension recorded being associated with less than 1 per cent shortening of the fiber.

REFRACTORY PERIOD. The possibility of the existence of a refractory period was investigated by stimulating with two equal shocks separated by a variable interval. The electrodes were made at least as long as the length of fiber under consideration (usually about 20 mm.). This procedure not only gave large responses for recording, since the entire length of fiber was involved, but provided a uniform field across the entire length of the fiber at each shock.

The stimulating equipment consisted of two condensers each discharging through its own gas triode (RCA 885). The interval between the shocks was controlled by a rheotome of the Keith Lucas type, two break keys of which controlled the starting of the condenser discharges by abruptly changing the grid voltage of the gas triodes in a positive direction. The stimuli were controlled in intensity by a potentiometer so arranged in

[3] Fresh, bright silver surfaces as well as freshly deposited silver chloride surfaces were found to polarize badly, yielding bubbles which were very troublesome. After use for some time in the serum-Ringer bath, the chlorided silver electrodes were found to be changed so that they no longer gave rise to bubbles. In this condition they could be used for months without requiring attention.

common in the anode circuits of the tubes that each discharge produced a transient current through it in the same direction. The time-constant of the discharge of a single condenser was measured as 1.1 msec.; the time-constant for simultaneous discharge of both condensers was 2.2 msec. The peak voltage across the potentiometer (or maximum peak voltage across the electrodes) was about 18 volts. Since about one-third of the distance between the electrodes was occupied by the fiber, the maximum voltage across the fiber was in the neighborhood of 6 volts. At threshold intensity the potential drop across the fiber was about 3 volts.

Unfortunately, the preparation underwent progressive changes in responsiveness; the size of response to constant stimuli increased for about ten minutes after the preparation was set up, then slowly decreased. This effect was found to depend in large part upon spontaneous changes in responsiveness and to a lesser extent upon the frequency of stimulation. The effect of the spontaneous changes upon the results here reported was minimized by the occasional interpolation of single constant stimuli as controls and by acceptance of the results only when these controls indicated a reasonably constant preparation. The effect of the increase in responsiveness existing for some seconds after a previous response was diminished by allowing periods of 30 seconds to 2 minutes to elapse between determinations.

If the peak tension developed under these conditions be plotted against the interval between stimuli it will be found that the peak tension decreases steadily with increasing intervals. The smooth curve relating the magnitude of the response and the interval between the two shocks (fig. 1) drops from its initial height when the shocks are synchronous, rapidly at first, then more and more slowly, approaching in about 30 msec. the level obtained in response to a single stimulus, and showing no tendency to rise again even for intervals longer than 100 msec. Long intervals between the first and the second shocks of the pair tend to produce reversible contractures following the second twitch in addition to the twitch response.

The curve shown in figure 1 is representative of all the experiments, which covered a range in temperature from 15°C. to 25°C., in which the controls indicated a reasonably constant response to constant stimuli. This type of curve was always obtained, independently of the order in which the points were taken.

The significance of these results may most readily be seen by a comparison with the results of a similar experiment upon a whole muscle. An entire sartorius muscle of the bullfrog was mounted so as to record isometric tension. The stimuli were applied through two fine silver wires placed at the pelvic extremity of the muscle. The stimuli were obtained from the same circuit used for stimulating the single fibers, and were of the same form and of essentially the same time-constant. When the peak

tensions were plotted against the interval between the two shocks in the same manner as in figure 1, the type of curve obtained was found to depend upon the intensity of the shocks. In the case of two maximal shocks (*A*, fig. 2) the response was independent of the interval and equal to the response to a single maximal shock for intervals up to 1.5 or 2.0 msec. For greater intervals the peak tension rose fairly sharply reaching a new level, sometimes poorly defined, after 5 to 10 msec. With two submaximal shocks (*B*, fig. 2) the peak tension reached a minimum earlier than 1 msec., thereafter rising as in curve *A*, although frequently showing a maximum before settling to the new high level.

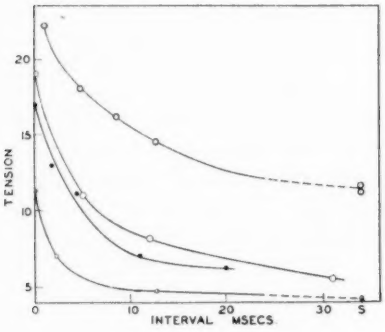

Fig. 1

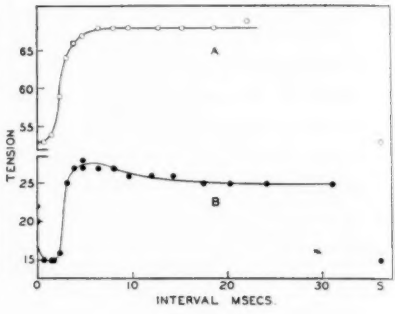

Fig. 2

Fig. 1. Peak tension developed by an isolated fiber, in arbitrary units, plotted against the interval between the two shocks which constituted the stimulus. Results from four fibers are shown. Points at extreme right represent the responses to single stimuli.

Fig. 2. Peak tension developed by an entire sartorius, in arbitrary units, plotted against the interval between the two shocks which constituted the stimulus. Both curves obtained from the same preparation. Points at extreme right represent the responses to single stimuli. Curve *A*: maximal shocks. Curve *B*: submaximal shocks.

The curves for a whole muscle shown in figure 2 are in substantial accord with the results of Hartree and Hill (1921), of Levinsohn (1910), and of Lippay (1935). The constancy of the response for short intervals in the case of maximal stimuli and the minimum in the response in the case of submaximal stimuli are due to the existence of an absolute refractory period. In curve *A*, at, say, 1 msec. separation between the shocks, the second shock, since it falls in the refractory period due to the first, can produce no effect, the response being due entirely to the first shock which is maximal. Thus the combined stimuli can produce no more response than a single maximal shock. With submaximal shocks (curve *B*) at the same

interval, the second shock likewise can have no effect upon the peak tension developed by the muscle. However, the combined submaximal stimuli produce less response at a 1 msec. interval than at zero interval due to the fact that, when the shocks are synchronous they constitute a stimulus nearer the maximal level so that some units are activated which are not excited by the separate stimuli. The subsequent sharp rise in both curves is evidently the sign of a relatively refractory period.

Since in dealing with the single fiber preparation stimulated with large electrodes there is no evidence of a well-defined maximal response to single shocks (Brown and Sichel, 1936), the results shown in figure 1 should perhaps be more properly compared with the curve for submaximal stimulation in figure 2 rather than with the curve for maximal stimulation. It

TABLE 1

EXPERIMENT	DISTANCE BETWEEN CATHODES	VOLTS	RESPONSE TENSION IN MM. DEFLECTION		
			To right electrode	To left electrode	To both electrodes simultaneously
	mm.				
III 10	3.5	18	0.0	0.0	0.5
11		26	0.0	2.5	4.0
12		34	1.5	6.0	7.5
VI 7	2.6	20	0.0	0.0	0.0
8		25	0.0	0.0	1.6
9		30	0.8	4.6	7.2
10		35	1.5	7.8	11.5
21	0.9	20	0.0	3.0	24.5
20		25	0.0	10.0	21.5
22		30	7.0	17.5	45.5

will be seen that there is no evidence for the existence of a refractory period in the single fiber preparation since a refractory period should cause the appearance of a minimum response as in figure 2. The second stimulus to an isolated length of fiber always affects the response, either by causing additional tension when the stimuli are close together or by maintaining tension when the stimuli are further separated. This is unlike whole muscle in which there is an interval during which a second stimulus does not affect the mechanical response.

SUMMATION: *Spatial summation.* The fact that the size of the responses is increased when large electrodes are used has been previously noted (Brown and Sichel, 1936). The effect of stimulation area was examined in experiments in which two cathodes at variable separation were used. The electrodes were of silver-silver chloride. The anode was a sheet about

0.2 mm. in thickness and 1 cm. wide placed close to the middle half of the length of the fiber. Two cathodes, each approximately 2 mm. wide were placed facing the opposite side of the fiber. These were arranged in parallel with suitable keys and potentiometers so that the fiber could be stimulated by each one separately, or by both simultaneously. The stimuli were direct current pulses, the intensity of which was variable, the duration being kept constant at 8 msec. by a Lucas spring rheotome.[4]

The results of representative experiments on spatial summation are summarized in table 1, and illustrative records are shown in figure 3. The table shows the results obtained by varying both the distance between the two cathodes and the intensities of the stimuli. From experiment III it will be seen that with a constant distance between the cathodes, the

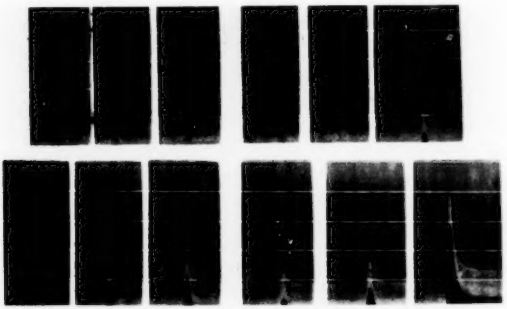

Fig. 3. Responses of a fiber to stimulation through two cathodes 1.5 mm. apart. In each group of three the first represents the response to stimulation through the right-hand cathode; the second through the left-hand cathode; the third through both cathodes simultaneously. In the group in the upper left-hand quadrant (*a*) the stimulus intensity was 20 units. In the upper right-hand group (*b*) the intensity was 25 units. In the lower left-hand group (*c*) the intensity was 30 units. In the lower right-hand group (*d*) the intensity was 35 units.

response to both stimuli simultaneously is always at least equal to and often greater than the sum of the responses to each stimulus alone. Further, this greater effectiveness of the simultaneous stimuli with reference to the sum of the individual responses is more pronounced at low voltages (and consequently at low levels of response). From experiment VI the additional fact is evident that with stimulus strengths that give comparable responses individually the effectiveness of the stimuli when simultaneous is relatively greater the smaller the distance between the cathodes.

The accuracy of the method employed in these experiments does not

[4] We are grateful to Dr. H. A. Charipper for making available for our use a rheotome from the apparatus stock of the Washington Square College, New York University.

warrant any detailed analysis. However, the results are qualitatively explicable in terms of summation of the electrical stimuli or of the consequent state of excitation of the contractile system. If the calculated potential difference across the fiber due to each cathode be plotted as a function of the distance along the fiber, a curve such as each of the lower ones shown in each part of figure 4 is obtained.[5] A maximum lies opposite the position of each cathode. Since the effective strength of the stimulus would be represented by the area under the curve and above the threshold line (dotted), the fiber should develop a tension which is a function of this

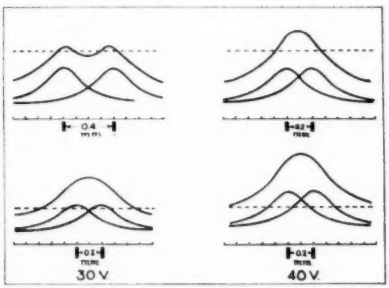

Fig. 4

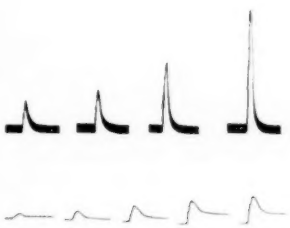

Fig. 5

Fig. 4. Representation of the potential drop across the fiber at various distances along the fiber away from the electrodes. The lower curves in each case represent the potential drop due to each cathode separately. The upper curve represents the drop due to both cathodes simultaneously. The broken line represents the threshold level. The upper left-hand figure (*a*) represents the case of two subthreshold stimuli, the cathodes being 0.4 mm. apart. In the three other cases the distance between the cathodes is 0.2 mm. In the upper right-hand figure (*b*) the individual intensities are the same as in (*a*). In the lower left-hand figure (*c*) the individual intensities are represented as being slightly above threshold. In the lower right-hand figure (*d*) the intensities are somewhat greater.

Fig. 5. Responses to two shocks separated by various time intervals. In the upper row the intervals are, from left to right: 31.0, 12.8, 5.0, 0.0 msec. Bottom row, left to right: single shock, 6.0, 3.5, 2.2, 0.0 msec.

area. Since potential differences may be added, the curve for both electrodes may be obtained by adding the ordinates due to each electrode alone. In *a*, (fig. 4) two subliminal stimuli are represented. When they are combined, an appreciable effective area above threshold is obtained. In *b*, stimuli of the same strength as used in *a*, are applied at half the distance apart and the total effective area of the two together is 8.7 times greater than in *a*. In *c* and *d*, stimuli above threshold are represented. With the lower intensity (30 V) the ratio of the area above threshold to the

[5] We are indebted to Dr. A. C. Burton for help in constructing these curves.

sum of such areas attributable to each electrode is 15.6 as compared with 5.1 at the higher voltage. The greater relative effectiveness of the stimuli at smaller distances apart and at lower voltages runs parallel with the changes in relative maximum tensions developed as a result of stimulation with similar variation in intensity and configuration of the stimulus.

We conclude that summation of the stimuli, or of the consequent local state of excitation, can account for the spatial summation observed in isolated lengths of muscle fiber.

Temporal summation. The results of the experiments on refractory period quoted above may be interpreted also in terms of temporal summation. Responses to two stimuli separated by various time intervals are illustrated by the results of representative experiments in figures 1 and 5. Seventeen experiments performed during the summer of 1937 and eighteen during the summer of 1938 are in general agreement with those shown. When the stimuli are synchronous, the tension developed is always more than double that due to either stimulus alone. Indeed, the apparent effect of the second stimulus may be several times that of the first. As the two shocks of the stimulus are separated, the additional effect of the second one declines rapidly so that with an interval of 2 to 4 msec. the peak tension in response to the two shocks is equal to the sum of the tensions in response to the two separately. With increasing intervals the total peak tension declines more slowly until with an interval of about 30 msec. the peak tension is very little higher than that developed in response to either stimulus alone. Examination of a fast record of the contraction, however, shows that the duration of the response is lengthened by a fusion of two twitch-like responses. If the second stimulus falls just after the peak of the twitch (40–50 msec.) the height of the second twitch is only slightly greater than that of the first. If the second stimulus comes later during relaxation it elicits approximately the same peak tension as the first and continues to do so at still longer intervals. As the experiment is repeated, the absolute level of the responses may change slowly, but the relation between the response and the interval between the shocks will remain as stated above. This variation in the absolute level is due to the fact mentioned above that the responsiveness of the preparation under the conditions of the experiment increases for about ten minutes to a maximum and then decreases slowly.

The findings in these experiments can be simply explained neither as the result of summation of stimuli nor as the result of summation of tension. Cathode ray oscillograph photographs of the stimuli show that the summation cannot be explained by an increase in the effective stimulus in this case as is possible with stimuli at two points on the fiber. Likewise, with longer intervals between shocks there is practically no summation of ten-

sion. Curves calculated on the assumption of simple summation of tension are of very different shape from those obtained experimentally. According to these calculated curves, if the summation were of tension it should still be evident for intervals between stimuli at least as long as the duration of the development of tension, whereas actually the maximum tension in response to the two shocks is no greater than to one alone for intervals somewhat shorter than this. As the time between shocks lengthens, the response to the second shock does not develop additional tension over and above that produced by the first, but does considerably increase the duration of the contraction, which fast recording shows clearly to be by a fusion of twitch-like responses.

The evidence indicates that the contractile mechanism of the isolated fiber is capable of temporal summation of an excitatory process in the sense in which that expression was used by Adrian and Lucas (1912).

Conclusions. Although in the intact nerve or muscle the refractory period is a consequence of the passage of a propagated disturbance (Adrian and Lucas, 1912) there is no reason to suppose that a refractory period necessarily must be preceded by a propagated disturbance. In the present case it seems justifiable to assume that we are dealing with a preparation which from its time relations of contraction has a normal contractile mechanism but in which contractions are graded and in which the conductile mechanism is not functioning (Brown and Sichel, 1930, 1936; Gelfan, 1930, 1933; Sichel and Prosser, 1935). We conclude then that the absence of any refractory period in the excitation of the isolated fiber is to be interpreted as meaning that associated with the contractile system there is no refractory period.

Since, as was shown by Adrian (1914), the all-or-nothing type of response is a logical consequence of the existence of a threshold of local excitatory change together with a refractory state, it is not surprising to find that responses may be graded in a preparation which has a threshold but does not have a refractory period. When the "local excitatory change" has reached the threshold level it will initiate a disturbance, of which in the isolated fiber the development of tension is the sign. However, additional stimulus, whether in the form of a larger single shock or of a second one following the first, by providing additional excitatory change, will still be effective in producing further tension since there is no refractory period to interfere.

The summation curve for the effect of two submaximal shocks indicates the time course of the excitatory process for the contractile system. It appears that this excitatory process declines from a maximum at or immediately after the moment of stimulation to practically zero within 30 to 40 msec. after the stimulus.

SUMMARY

1. The contractile system in isolated lengths of skeletal muscle fiber (frog) was investigated by stimulating with pairs of condenser discharges applied across the entire length of the piece of fiber. To determine whether the response is or is not associated with a refractory period and whether it shows temporal summation, the interval between the shocks was varied.

2. It was found that when the shocks were synchronous, the response was larger than the sum of responses to the two shocks separately. The response then decreased as the interval between the shocks increased.

3. By comparison with the behavior of the entire sartorius stimulated with similar pairs of shocks, it is concluded that the graded responses of the isolated length of fiber are not associated with a refractory period.

4. The isolated length of fiber shows a spatial summation in that the response to simultaneous stimulation at two points along the fiber is larger than the sum of the responses to the two stimuli separately. The relative amount of spatial summation is greater at low voltages and at small distances between the stimulating cathodes.

5. It is concluded that stimulation by shocks at two points on a fiber results in spatial summation of stimuli while stimulation by shocks separated in time indicates temporal summation of an excitatory process which leads to the development of tension and which lasts for approximately 30 msec.

6. The existence of graded responses in the isolated length of fiber is shown to be a consequence of the absence of a refractory period.

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A QUANTITATIVE STUDY OF OESTROGEN-PROGESTERONE INTERACTION IN THE FORMATION OF PLACENTOMATA IN THE CASTRATE RAT[1]

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There have been several attempts made in the past to produce placentomata in castrate rats. Weichert (16) was able to produce placentomata in castrate rats and guinea pigs with the use of corpus luteum preparations. Nelson and Pfiffner (9) confirmed these findings in the rat, and Goldstein and Tatelbaum (6) in the guinea pig. The latter workers showed that the reaction could be obtained with the corpus luteum hormone but not with oestrin. Shelesnyak (14) after appropriate oestrin treatment followed by corpus luteum treatment was able to produce the reaction in 19 day old female rats.

It appeared to us to be of interest to make a quantitative study of this reaction involving the following aspects: 1, the effect on placentoma size of *a*, different doses of progesterone; *b*, constant doses of progesterone given in combination with varying doses of oestradiol; *c*, doubling the number of injections of a constant dose of progesterone or a progesterone-oestradiol combination; 2, the comparison of the effect of a given dose of progesterone on placentoma size in *a*, rats hypophysectomized with ovaries intact; *b*, rats both hypophysectomized and castrated, and *c*, castrate rats with hypophysis intact.

MATERIAL AND METHODS. One hundred and eighty-two young adult female rats, after having shown regular cycles, as determined by the vaginal smear technique, were made pseudopregnant by mechanical stimulation of the cervix. On the 4th day of pseudopregnancy they were castrated, and the uterus traumatized by passing a needle into the lumen and piercing the antimesometrial wall in several places along its length. They were then injected with a given progesterone dose, or with a progesterone-oestradiol combination from the 4th day to the 8th day inclusive, and killed on the 9th. At autopsy the uteri were dissected free of the mesometrium and any adherent fat, weighed on an analytical balance and

[1] This work was supported in part by a grant-in-aid from the Wisconsin Alumni Research Foundation, and by assistance supplied by the W. P. A. University of Wisconsin Natural Science Project.

fixed in Bouin's fluid. At some time after complete fixation, usually not earlier than one week, the uteri were removed from the fixative and the placentomata were measured along the antimesometrial-mesometrial axis at their widest diameter. If the reaction was negative, several measurements were taken at random from both horns. The uteri were measured to within one-tenth of a millimeter.

The animals were divided into three main groups according to the total dose of progesterone[2] given. These were 1.5, 3.0 and 6.0 Rb.U. of the non-crystalline or 1.5 and 3.0 mgm. of the crystalline progesterone. Each of these groups was further subdivided according to the number of injections the animal received per day. Thus, 60 rats received a total dose of 1.5 Rb.U., 86 rats received 3.0 Rb.U. and 36 rats received 6.0 Rb.U. In the 1.5 Rb.U. series, 15 rats received the hormone or hormones in single daily injections, and 45 in twice daily injections. In the 3.0 Rb.U. series, 52 received the hormone in single daily injections and 34 in twice daily injections, and in the 6.0 Rb.U. series 24 received the hormone in single daily injections and 12 in twice daily injections.[3]

Each of these groups was again sudivided according to the total dose of oestradiol added to the progesterone. The oestradiol level ranged from 0.075 to 9.0 gamma total dose. The number of animals in each of the final divisions varied from 3 to 13. The distribution of the animals according to total dose of progesterone, number of daily injections and amount of oestradiol received can be seen in table 1.

Corn oil was used as the solvent in all cases, and where a progesterone-oestradiol combination was used, the oestrogen was added directly to the solution. The volume was 0.10 cc. per injection throughout, animals in

[2] The progesterone used was from two sources. The first, a non-crystalline synthetic preparation, was made from cholesterol by one of us (M. A. S.) (15) and was of two kinds. One was probably free of oestrogens, as the method of preparation excluded their formation, and was relatively free of androgens. That is, the equivalent of 10 Rb. U. injected into immature 40 day old male rats for a period of 5 days caused the seminal vesicle weight to increase from 16 mgm. (control weight) to 28 mgm. The other preparation was also probably free of oestrogens, for the same reason, but had a higher androgen content. The equivalent of 10 Rb. U. of this preparation injected as before increased the seminal vesicle weight to 149 mgm. (15). Since the results obtained with the two synthetic non-crystalline preparations were the same they will be considered as one preparation. The other source of progesterone was a crystalline product of the Schering Corporation, Bloomfield, N. J., which was donated through the courtesy of Dr. Erwin Schwenk, to whom we are also indebted for the oestradiol used in this study. There were some differences between the effect of the crystalline and non-crystalline progesterones which are brought out in "Results."

[3] The symbols $\times 1$ and $\times 2$ will be used throughout to represent the number of daily injections. Thus $\times 1$ will indicate single daily and $\times 2$ twice daily injections.

TABLE 1

The effect of progesterone dose, progesterone-oestradiol combinations and divided dosage of placentomata size

Rb. U.		OESTRADIOL†	EXPERIMENTAL GROUP	NUMBER RATS	NUMBER POSITIVE CASES	AVERAGE NUMBER PLACENTOMATA PER RAT	TOTAL NUMBER PLACENTOMATA IN GROUP	MEAN PLACENTOMATA DIAMETERS	EXPERIMENTAL GROUPS TESTED	PROBABILITIES
		gamma						*mm.*		
		0.00	1	3	0	0.0	00	3.30[n]		
		0.15	2	3	0	0.0	00	2.58[n]		
	×1*	0.30	3	3	0	0.0	00	2.67[n]		
		0.75	4	3	0	0.0	00	2.30[n]		
1.5†		1.50	5	3	0	0.0	00	2.12[n]		
		0.00	6	8	7	5.1	36	4.54	6, 8	>0.05
		0.075	7	8	5	7.6	38	4.65		
		0.15	8	11	8	11.1	89	5.43	6, 19	<0.01
	×2*	0.30	9	5	4	8.5	34	4.88		
		0.90	10	5	3	7.7	23	5.09		
		1.50	11	8	2	5.0	10	4.43[p] 3.69[p,n]		
		0.00	12	13	13	8.3[a]	66	4.98	12, 13	<0.05 >0.01
		0.15	13	7	6	11.2	71	5.85		
		0.30	14	9	9	8.1[b]	65	5.69	12, 26	0.01
		0.90	15	6	6	11.0[c]	55	5.67		
	×1	1.50	16	5	4	10.5	42	4.96	12, 19	>0.05
		2.10	17	5	5	12.2	61	4.87		
		3.00	18	7	3	10.3	31	4.40[p] 3.93[p,n]		
3.0										
		0.00	19	6	5	11.0	55	5.49		
		0.15	20	5	5	11.6	58	5.48	19, 21	<0.05 >0.01
		0.60	21	5	5	11.6	58	6.45		
		0.90	22	5	5	10.4	52	6.04		
	×2	1.50	23	5	5	12.0	60	5.70		
		3.00	24	4	4	9.3	37	4.85		
		6.00	25	4	2	6.5	13	3.68[p] 3.35[p,n]		
		0.00	26	6	4	9.3	37	5.60	26, 28	0.01
		0.30	27	4	3	13.0	39	6.27	26, 32	0.01
		0.60	28	4	4	13.3	53	7.14		
	×1	1.50	29	4	4	11.7	47	6.21		
		6.00	30	3	3	9.0	27	4.89		
6.0		9.00	31	3	3	7.0	21	4.76		
		0.00	32	5	4	14.8	59	6.12		
	×2	6.00	33	4	3	10.0	30	5.17	19, 32	<0.05 >0.01
		9.00	34	3	3	10.0	30	5.17		

[a] Average of 8 rats; [b] average of 8 rats; [c] average of 5 rats; [p] average of measurements on positive cases only; [n] average of measurements on negative cases only.

* ×1, single daily injections; ×2, twice daily injections.

† The progesterone and oestradiol doses shown are the total doses.

the × 1 series receiving a total volume of 0.50 cc., and those in the × 2 series receiving a total volume of 1.0 cc.

For the study of the effect of hypophysectomy on placentomata formation, 7 rats were hypophysectomized on the 4th day of pseudopregnancy, but the ovaries left intact, and 6 rats were both hypophysectomized and castrated on the 4th day of pseudopregnancy. Trauma of the uterus was done in both groups as described for the other experiments. They were injected with the same dose of progesterone (3.0 Rb.U. × 2) and autopsied on the 9th day.

RESULTS. The mean placentoma diameter for all the rats in a particular group was obtained by dividing the sum of the values of all the placentomata diameters in the group by the total number of placentomata in the group. In testing the significance of the difference between group means, the following formula was used:

$$t = \frac{X_1 - X_2}{\sqrt{\frac{\xi d^2_{B_1} + \xi d^2_{B_2}}{n_1 + n_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

where X_1 is the mean of the first group, X_2 is the mean of the second group, n is the number of animals per group, N is the number of observations per group (total number of placentomata per group) and $\xi d^2_{B_1}$ is derived from the following formula:

$$\xi d^2_{B_1} = \xi \frac{(\xi x_{1i})^2}{K_{1i}} - \frac{(\xi x_1)^2}{N_1}$$

where x_{1i} is the value of an individual observation (a single placentoma diameter), ξx_{1i} the sum of the values of the observations per rat, K_{1i} the number of observations per rat, ξx_1 the sum of the values of all the observations, and N_1 the total number of observations, in group 1. Similarly, $\xi d^2_{B_2}$ for group 2. The probability of a mean difference $(X_1 - X_2)$ of this size or larger occurring by chance is obtained by reference to a table of t values, for instance, table XIII, Davenport and Ekas, 4th ed., John Wiley and Sons, N. Y. A probability of greater than 0.05 was not considered as being statistically significant.

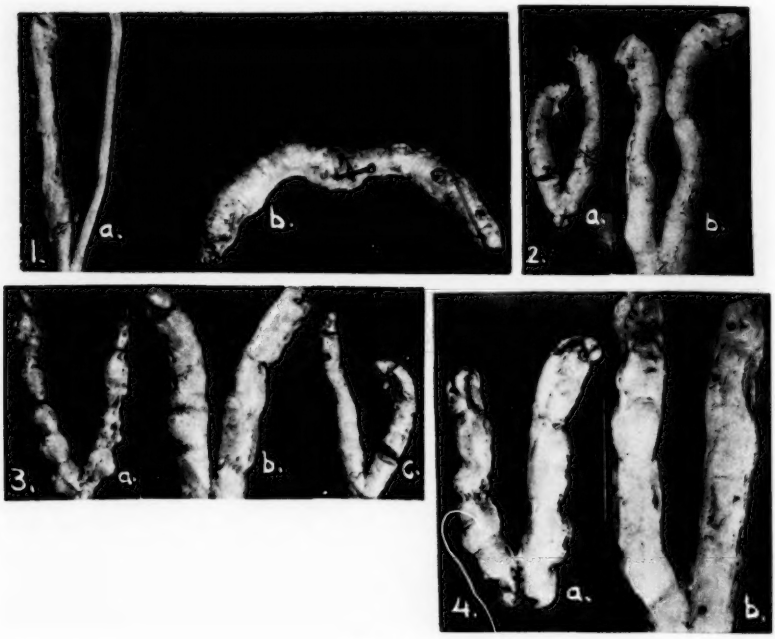
The total number of animals used, the number of animals reacting positively, the number of placentomata per group, the mean placentoma diameters and the probabilities are shown in table 1. Of the 182 animals treated, 137 showed the presence of placentomata at autopsy. It can be seen that of the 45 negative animals, 27 or 60 per cent are distributed in the 8 experimental groups, nos. 1–5, 11, 18 and 25, comprising 34 animals, in which low progesterone dosage or relatively high oestradiol dosage was employed, while 18 negative animals or 40 per cent of the total are distributed among 12 random experimental groups, nos. 6–10, 13, 16, 19, 26, 27, 32 and 33, comprising 74 animals. The percentage of negative cases in the former groups is thus 79 per cent, while in the latter groups it is 24.3 per cent. The distribution of the negative cases can thus be seen to be associated primarily with low progesterone dosage and with progesterone-oestradiol combinations of relatively high oestradiol content.

1. *The effect of progesterone, progesterone-oestradiol combinations and divided dosage on placentomata size.* a. *Progesterone only.* With the lowest progesterone dose used, 1.5 Rb.U × 1, the reaction failed to occur. Increasing the dose to 3.0 Rb.U. and 6.0 Rb.U. resulted in positive reactions, the size of the placentomata increasing with the dose. Thus, with 3.0 Rb.U. × 1 the mean placentoma diameter was 4.98 mm., and with 6.0 Rb.U. × 1 it was 5.6 mm. In the twice daily injection series the reaction occurred at all three dose levels, the means being 4.54 mm., 5.49 mm., and 6.12 mm. respectively. The differences between the means in both the × 1 and × 2 series are statistically significant (table 1).

b. *Divided dosage.* The most striking effect of this procedure was seen in the 1.5 Rb.U. series. As already mentioned, this dose of progesterone given in single daily injections failed to elicit the reaction, but when the injections were given twice daily the reaction was positive (fig. 1). With the other progesterone doses used, such striking changes did not take place, although there were differences between the means in favor of those groups receiving the divided dosage. Thus with 3.0 Rb.U. × 1 the mean was 4.98 mm., and with the same dose × 2 the mean was 5.49 mm. With 6.0 Rb.U. × 1 the mean was 5.6 mm., with the same dose × 2 it was 6.12 mm. (table 1). While the differences between the × 1 and × 2 means was the same for both of the two higher progesterone dose groups, a statistically significant difference (P = 0.01, table 1) could be demonstrated only between the 6.0 Rb.U. × 1 and 6.0 Rb.U. × 2 means.

c. *Progesterone oestradiol combinations.* In four of the series studied (1.5 Rb.U. × 2, 3.0 Rb.U. × 1 and × 2, and 6.0 Rb.U. × 1) both an augmentative and an inhibiting[1] effect of the action of progesterone by oestradiol was seen. That is, with increasing levels of oestrogen, the effect of the progesterone was augmented by the lower doses up to an optimum level and inhibited by the higher doses. Thus in the 1.5 Rb.U. × 2 series the addition of 0.15 gamma of oestradiol increased the mean placentoma diameter from 4.54 mm. to 5.43 mm., but with the addition of 1.5 gamma of oestradiol the reaction occurred in only 2 out of 8 rats, and in these was of such small size that the presence of placentomata could be demonstrated only microscopically. With 3.0 Rb.U. × 1 definite augmentation was seen with the addition of 0.15 gamma of oestradiol, while with 3.0 Rb.U. × 2 the augmenting level was 0.60 gamma of oestradiol. The inhibiting levels of oestradiol were also not the same in these two latter series, 3.0 gamma of oestradiol inhibiting 3.0 Rb.U. × 1 while 6.0 gamma of oestradiol were needed to inhibit 3.0 Rb.U. × 2. In the

[1] The term "inhibition" as used in this paper implies not only the complete prevention of the formation of placentomata but also the formation of placentomata smaller than those obtained with progesterone only, or smaller than those obtained with progesterone in combination with augmenting doses of oestradiol.



Figs. 1–4

Fig. 1. The effect of divided dosage. These uteri are from rats which received a total dose of 1.5 Rb. U. of progesterone, *a*, in single daily injections; *b*, in twice daily injections. *a* is a negative reaction, *b* shows the presence of placentomata (natural size).

Fig. 2. The effect of divided dosage. These uteri are from rats which received a total dose of 3.0 Rb. U. of progesterone plus 3.0 gamma of oestradiol, *a*, in single daily injections; *b*, in twice daily injections. The reaction has been inhibited in *a*, but has occurred in *b* (× 5/6).

Fig. 3. The augmentative and inhibiting effect of oestradiol on progesterone action. These uteri are from rats which received a total dose of 3.0 Rb. U. of progesterone in single daily injections, *a*, with no additional oestrogen; *b*, plus 0.15 gamma oestradiol; *c*, plus 3.0 gamma oestradiol. The effect of the oestradiol is seen in an augmentation of the reaction in *b*, and an inhibition of the reaction in *c* (× 7/8).

Fig. 4. The augmentative effect of oestradiol on progesterone action. These uteri are from rats which received a total dose of 6.0 Rb. U. of progesterone in single daily injections, *a*, with no additional oestrogen; *b*, plus 0.60 gamma oestradiol. The reaction is definitely augmented in *b* (natural size).

6.0 Rb.U. × 1 series the best augmentation took place at the 0.60 gamma oestradiol dose while with as much as 9.0 gamma of oestradiol the reaction was not completely inhibited although the reduction in size from 5.6 mm. to 4.76 mm. is apparent (table 1, figs. 2, 3, 4). When tests for statistical

significance were carried out for differences between the means obtained with the progesterone only and those obtained with progesterone plus the augmenting level of oestradiol, it was found that these differences were significant in all but the 1.5 Rb.U. × 2 series.

The possibility that the augmenting effect of oestradiol was due to increase in size of elements other than decidual tissue was tested in several cases by microscopic measurements of the decidual tissue alone. The results obtained by this method checked closely with those obtained by gross measurements and showed clearly that the increase in decidual tissue was responsible for the increase in size.

In the 1.5 Rb.U. × 1 series no synergistic effect of the addition of oestradiol was seen although doses ranging from 0.15 gamma to 1.5 gamma were used. In all cases the reaction was negative. In the 6.0 Rb.U. × 2 series only two oestradiol doses were used, 6.0 and 9.0 gamma. Both of these doses partially inhibited the action of the progesterone to the same extent, reducing the mean placentoma size from 6.12 mm. to 5.17 mm.

In addition to the differences in size between placentomata in the high oestradiol groups and those in the low oestradiol and progesterone only groups, a structural difference was brought out after histological study. The histological appearance of placentomata has been described before (8, 12) and need not be gone into in any detail here. A few of the important features, however, deserve mention. The stroma is composed almost entirely of decidual tissue which is differentiated into two types. The first, the antimesometrial decidua, is made up of large cells, whose cytoplasm shows a strong affinity for eosin. Very often these cells are binucleate. This tissue extends from the lumen almost to the muscularis, and fills nearly the whole of the antimesometrial half of the uterus. The other type of decidual tissue is made up of smaller, vacuolated cells, arranged in what appear to be connecting islands of tissue. This appearance is due to the enlargement of the capillaries in this region into sinusoids, resulting in a spreading apart of large groups of cells. This tissue always lies in the mesometrial half of the uterus. Krehbiehl (8) has shown that these two areas can be further differentiated from each other in that the mesometrial decidua always contains glycogen in very appreciable quantities, whereas the antimesometrial decidua never contains glycogen after the first few days of its appearance, but becomes typically a fat-laden tissue.

The most important facts brought out by a study of the placentomata obtained in our study may be stated as follows: there was a change in the relative proportions of the mesometrial to the antimesometrial decidua depending on the level of oestradiol used. In the 6.0 Rb.U. × 1 series, with the addition of oestradiol doses up to 1.5 gamma, there was little change in the proportions of these two areas, the mesometrial decidua comprising between 33 per cent and 50 per cent of the total decidual area

in cross section. When 6.0 gamma of oestradiol were used, the mesometrial area was only 15 per cent and with 9.0 gamma only about 10 per cent of the total decidual area. When the latter two dosage combinations were given in twice daily injections, the placentomata produced consisted of only antimesometrial decidua in 5 out of 6 animals, while in the 6th the mesometrial decidua was about 33 per cent of the total decidual area (fig. 5). The same effect of high oestradiol dose in proportion to progesterone dose

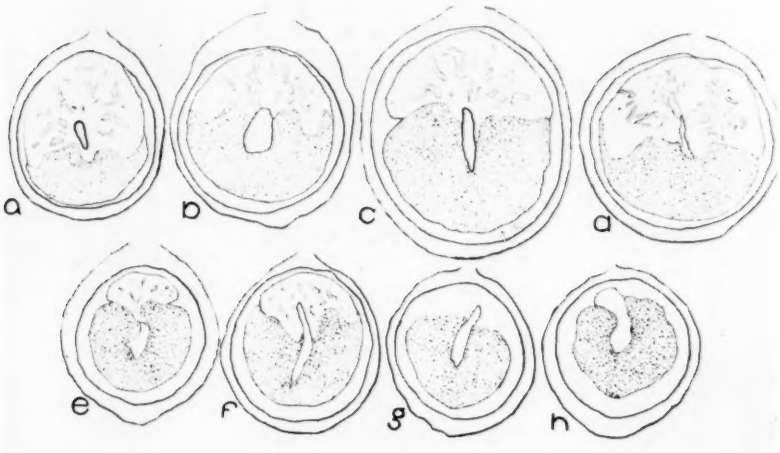


Fig. 5. Semischematic cross-sections through placentomata. The total progesterone dose in all cases was 6.0 Rb. U. The oestradiol doses were *a*, no oestradiol; *b*, 0.30 gamma; *c*, 0.60 gamma; *d*, 1.5 gamma; *e*, 6.0 gamma; *f*, 9.0 gamma; *g*, 6.0 gamma; *h*, 9.0 gamma. The injections were single daily in all cases except *g* and *h*, where they were twice daily. The light area with irregular spaces represents the mesometrial, the stippled area the antimesometrial portions of the decidual area. The two outermost lines enclose the area occupied by muscle. The central space within the decidua is the lumen. The mesometrial portion of the decidual area can be seen to be practically unchanged in relative amount until 6.0 and 9.0 gamma of oestradiol are added to the progesterone, when this area either disappears or is definitely reduced in amount (× 4).

was seen in the study of the 1.5 Rb.U. × 2, and 3.0 Rb.U. × 1 and × 2 series, and it may be said as an almost general rule that when placentomata appear with progesterone-oestradiol combinations of relatively high oestradiol content, they will be composed predominantly of antimesometrial decidua, and that the mesometrial decidua will be absent, or present in only small amounts.

2. *The effect of hypophysectomy on placentoma size.* As can be seen from table 2, the removal of the pituitary with the ovaries left intact in rats

given 3.0 Rb.U. of progesterone × 2 resulted in the formation of placentomata of extremely large size. The mean placentoma diameter in these rats was 7.84 mm., an increase of 2.35 mm. over that obtained with the same progesterone dose in castrate rats with pituitary intact. When, however, the ovaries were removed as well as the pituitary, the mean diameter decreased to 6.04 mm. There was no statistically significant difference between this latter mean and that obtained in the castrate rat with pituitary intact and given the same progesterone treatment.

Only in two series was it possible to compare the effects of the two types of progesterones used, since the supply of the crystalline material was limited. This was done in the 1.5 mgm. × 2 series and in the 3.0 mgm. × 1 series. In the former, three dosages were used, namely, progesterone only, and with 0.15 gamma and 1.5 gamma of oestradiol. The results obtained paralleled those obtained with the non-crystalline material almost exactly

TABLE 2

The relation between placentomata size and the presence or absence of the ovaries and pituitary in rats receiving a constant dose of progesterone (3.0 Rb. U. × 2)

	Ov-ect. AP	Ov-ect. AP-ect.	OAP-ect.
Number of rats	5	6	7
Total number placentomata in group	55	42	48
Mean placentoma diameter (mm.)	5.49	6.04	7.84
Groups tested	Ov-ect. AP, Ov-ect.AP-ect.		Ov-ect. AP, OAP-ect.
Probabilities	>0.05		<0.01

O, ovaries intact; Ov-ect., castrate; AP, pituitary intact; AP-ect., hypophysectomized.

but at a slightly lower level. In the 3.0 mgm. series the dosages were progesterone only, and with 0.15 gamma and 3.0 gamma of oestradiol. One of the animals on the 0.15 gamma dose was negative, and the other had only a microscopic placentoma. Those in the progesterone only group were positive, and those in the high oestradiol group negative. It is probable that the animals in the 0.15 gamma oestradiol group were in the nature of "misses." The preliminary work on this study was done with crystalline material using 3.0 mgm. alone and 3.0 mgm. plus 0.30 gamma of oestradiol. Both of these groups reacted positively, 8 rats being used. The size of these placentomata were of the same order as those obtained with the non-crystalline material.

The placentomata obtained in our study were smaller than those formed in normal intact pseudopregnant rats, with certain exceptions. In the 6.0 Rb.U. × 1 series, the addition of 0.30, 0.60 and 1.5 gamma of oestradiol

produced placentomata of normal size or larger, while with 3.0 Rb.U. × 2 plus 0.60 gamma they were of normal size.

Discussion. The development in the rat, and the maintenance in the rabbit of the progestational picture in the uterus has been shown to depend on the simultaneous action of progesterone and oestrogen (13, 7, 2). Allen (2) also showed that the way in which the uterus of the rabbit reacts to pituitrin during the course of pregnancy depends upon a progesterone-oestrogen combination and cannot be duplicated by progesterone alone. The augmentation of progesterone action has been shown to occur by Pincus (10) in the growth of the rabbit blastocyst and the development of progestational proliferation in the rabbit uterus. Pincus (10) believes that this augmentation might be due to the saturation by the oestrogens of the enzyme system which destroys both hormones, the end result being that the destruction of the progesterone is slowed up and its action consequently prolonged. Another possible explanation for the enhancing effect of oestradiol might be derived from the property the latter has of increasing the blood supply to the uterus (3, 11) thus making the progesterone available to more of the stromal tissue.

More extensive data, however, concerning augmentation of progesterone by other compounds of steroid nature, or by oestrogens of non-steroid nature, must be obtained before these or any other theories of augmentation of progesterone can be properly evaluated.

A question of interest is that of whether oestrin plays a part in the normal physiology of implantation. The fact that oestrin has been reported as occurring in appreciable quantities in extracts of corpora lutea (1, 5) may mean that this synergism is a normal process. It should be understood at this point, however, that a physiologically significant difference between placentomata formed under the influence of progesterone only, and those formed under the influence of progesterone-oestradiol combinations has not been demonstrated, although a statistically significant difference has. Until it can be shown that implantation proceeds normally only when the two hormones are present, the synergistic action of oestradiol described in this study must be considered as only one of several possible factors involved in the process.

The effect of the increasing quantities of oestradiol when given with the progesterone on the size of the glycogen bearing area of the placentoma suggests that oestrogen may prevent the deposition of glycogen in this part, labilize it so that it becomes available to the embryo, or delay the time of its appearance. The end result of any of these effects would be a decrease in size of this area. Certain data bearing on this general problem have been obtained by Rothchild and McShan (unpublished). These data show that the glycogen of placentomata is very stable, and not easily influenced by such factors as starvation, injection of adrenalin

or hypophysectomy. Studies are now being undertaken to determine what effect oestrogens have on the stability of glycogen in the placentoma. These studies may yield data which will be of value in explaining the deleterious effect of oestrogen on the life of the embryo.

The effects obtained in the hypophysectomized rats can probably be explained by the assumption that the progesterone was absorbed at a slower rate due to a lowering of the metabolism in these rats and that it was augmented, after absorption, by the oestrogens still being secreted by the ovaries.

Since the placentomata in these studies were formed in uteri which had not only been sensitized but also prepared for trauma by oestrogen and progesterone secreted by their own ovaries, the question arises as to whether it is possible to form placentomata in uteri which have been artificially prepared from the first. This we can partially answer at present. We have found in long time castrates that it is not only possible to form placentomata after appropriate pretreatment with oestrin, but also, if sufficient progesterone is given, to form them without any pretreatment of any kind.

While this was being written, a similar study by Astwood (4) appeared. He states that his attempts to produce placentomata in long time castrate rats gave inconsistent results, but that good success was obtained in rats castrated on the 4th day of pseudopregnancy and injected with progesterone for 3 days after. He was unable to show the synergistic effect of oestradiol, however, although an inhibitory effect was seen. It is possible that no augmentation was seen because of two factors: 1, the small number of animals on any particular dose, and 2, the fact that they were killed 3 days after trauma, at which time, as Krehbiehl (8) has shown, the mesometrial decidua is just starting to form in normal intact pseudopregnant animals.

We wish to express our thanks to Dr. A. B. Chapman of the Department of Genetics, University of Wisconsin, for his help in the statistical treatment of the data.

SUMMARY

1. One hundred and eighty-two female rats were treated with progesterone, or progesterone-oestradiol combinations from the 4th to the 8th day of pseudopregnancy inclusive, after being castrated and the uterus traumatized on the 4th day. The injections were either single or twice daily.

2. The size of the placentomata formed under these conditions varied directly with the progesterone dose and the number of daily injections, i.e., the larger the progesterone dose, the larger the placentomata; the placentomata obtained with the same progesterone dose when given in single or twice daily injections were larger with the latter method.

3. The addition of oestradiol in varying doses to a constant progesterone dose caused increase in the size of the placentomata until an optimal level of oestradiol was reached. Further increase in oestradiol level resulted in the formation of smaller placentomata and finally in the complete inhibition of the reaction.

4. The formation of placentomata of normal histological appearance was compatible with all the oestradiol levels used except the higher ones. With the use of high oestradiol doses the reduction in size of the placentomata was associated with reduction in amount or disappearance of the mesometrial portion of the decidua.

5. Placentomata could be formed in the absence of the pituitary gland, after appropriate progesterone treatment.

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STUDIES ON WATER BALANCE IN THE ALARM REACTION

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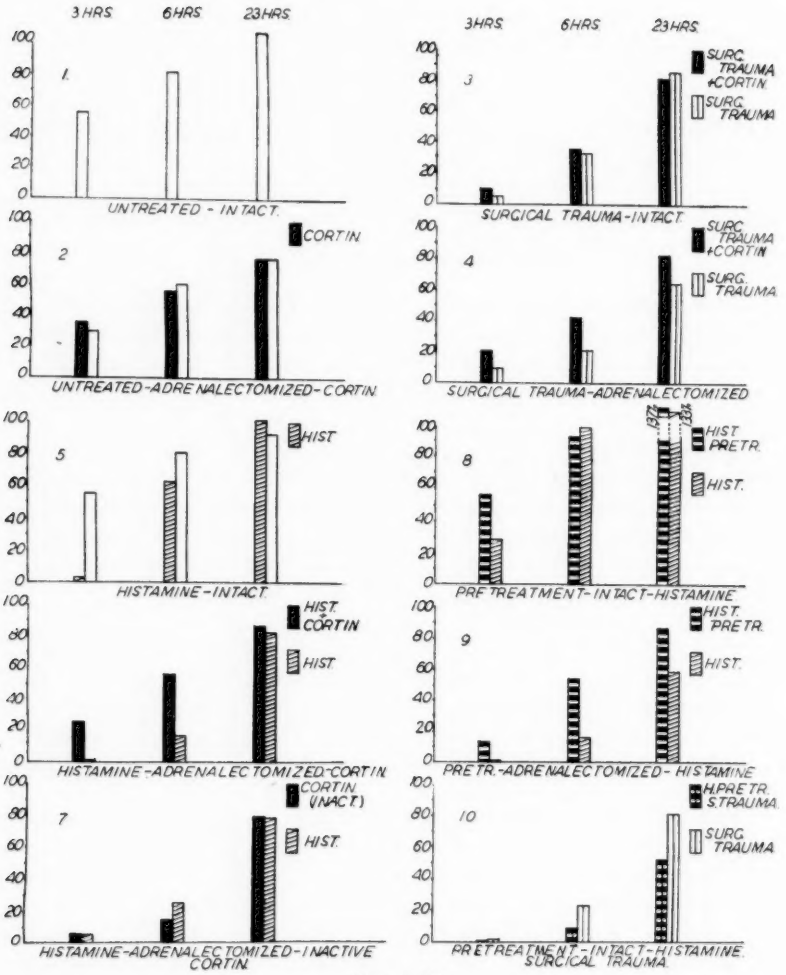
A syndrome has been described by Selye (1) which appears when an organism is submitted to a severe non-specific injury, whether it be cold, trauma, excessive muscular exercise, spinal shock, or nocuous drugs. In the first stage of this syndrome called the "alarm reaction" there is among other symptoms and morphological changes a marked tendency to accumulate fluid in the extra-vascular spaces (edema formation) and to the formation of pleural and peritoneal transudates. The degree of the edema depends on the degree of damage and upon the amount of water available to the organism. However, even if the damaging agent is continued the edema disappears after a varying period usually of from two to four days. The adrenal cortex hypertrophies at about the same time.

The present study deals with the rate of urinary secretion of intravenously injected 0.85 per cent NaCl solution after manipulation of the stomach and intestines or after injection of histamine in intact and adrenalectomized rats. The effect of cortin and of pretreatment with histamine on these phenomena has been studied. A preliminary report of this investigation was presented at a meeting of the Canadian Physiological Society (2).

METHODS. Hooded rats, about three months of age, weighing between 150–200 grams, were used. They were fed on a diet of purina. Both males and females were used and no difference was observed in the responses of the two sexes. The animals were anesthetized with ether and then given one-tenth of the body weight of 0.85 per cent sodium chloride solution intravenously into the internal jugular vein. In most cases the animals were fasted for fifteen hours before the experiment was begun. Adrenalectomized animals were given saline to drink from the time of the adrenalectomy until the time of the experiment, and were in good condition. Intact animals received water without the addition of salt. During the time of the experiment the animals received neither food nor drink. They were kept in round cages which were suspended over funnels and the urine was collected in graduated cylinders. The urine volume was measured three, six and twenty-three hours after the injection. In the figures the measurements are expressed as the percentage of the volume



Figs. 1–10

The effect of various damaging agents upon urinary excretion of intravenously injected saline in the rat, 3, 6 and 23 hours after the injection.

Fig. 1. Normal intact animals.

Fig. 2. Untreated adrenalectomized animals. The black columns show the effect of cortin treatment.

Fig. 3. Intact animals with surgical trauma. The black columns show the effect of cortin treatment.

Fig. 4. Adrenalectomized animals with surgical trauma. The black columns show the effect of cortin treatment.

Fig. 5. Intact animals treated with 15 mgm. histamine. The blank columns show the response in the untreated controls.

injected which was excreted. In some instances chloride by the method of Van Slyke (3) and sodium determinations by the method of Butler and Tuthill (4) on the urine were also carried out, and the results are expressed in the same way as for the water. In the experiments where cortin was injected, since the cortin contained 0.85 per cent NaCl, the volume of the extract injected was used to replace part of the saline, the total amount injected always being one-tenth of the body weight. The extract employed[1] contained 10 Swingle Dog Units per cubic centimeter.

EXPERIMENTAL RESULTS. The excretion of water after giving eight normal intact animals saline intravenously, as described above, is shown in figure 1. It is 55 per cent in three hours, 81 per cent in six hours, and 104 per cent in twenty-three hours. The chloride excretion was 105 per cent in twenty-three hours.

Figure 2 shows the results of the same experiment using eight adrenalectomized animals. It is seen that a lower percentage of the injected saline was excreted in this group than in the intact animals. The black columns in this figure show the lack of effect of 2 cc. of cortin given intravenously to a group of four animals at the time of the experiment. The four cortin treated animals excreted fluid at the rate of 35 per cent in three hours, 54 per cent in six hours and 76 per cent in twenty-three hours. The corresponding values for the nontreated controls were 29 per cent in three hours, 60 per cent in six hours, and 76 per cent in twenty-three hours.

The surgical trauma was produced by opening the abdomen and exteriorizing the intestines for a minute and a half. The intestines were gently handled, whilst the stomach was pressed firmly and as uniformly as possible between the thumb and forefinger. In some instances the trauma was inflicted on the stomach by pressing it with an artery forceps, once, twice or three times depending upon the severity of the trauma desired. In figure 3 is shown the effect of such trauma on decreasing the rate of water excretion in a group of six intact animals as compared with the untreated

Fig. 6. Adrenalectomized animals with 1.5 mgm. histamine. The black columns show the effect of 2 cc. of cortin at the time of experiment.

Fig. 7. Adrenalectomized animals with 1.5 mgm. of histamine. The black columns show the effect of inactivated cortin, 2 cc. with the injected fluid.

Fig. 8. Intact animals pretreated with histamine and injected with 40 mgm. of histamine. The cross hatched columns show the result of this dose of histamine on the not pretreated controls.

Fig. 9. Adrenalectomized animals with histamine pretreatment and 1.5 mgm. of histamine. The cross hatched columns show the effect of this dose on not pretreated animals.

Fig. 10. Intact animals pretreated with histamine and surgically traumatised. The center lined columns show the response to this trauma of non-pretreated controls.

[1] We wish to thank Dr. E. W. McHenry and the Connaught Laboratories, Toronto, for their generosity in supplying this material.

controls in figure 1. The black columns show the lack of effect of 2 cc. of cortical extract in a group of six animals similarly traumatized. Five cubic centimeters of cortical extract given at the time of the experiment, and in other groups daily injections of 2 cc. of cortical extract given subcutaneously for three or four days, as well as 2 cc. intravenously at the time of the experiment did not increase the rate of water excretion in surgically traumatized intact animals.

In contrast with this, the effect of cortical extract in increasing the rate of water excretion of traumatized adrenalectomized animals is shown in figure 4. In this experiment there were 20 control and 20 cortin treated animals. In the control group in figure 4 the percentage of water excretion in the adrenalectomized surgically traumatized animals is obviously less than that in the untreated adrenalectomized group in figure 2, and with the exception of the three hour period, also less than that of the control group of figure 3 in spite of the fact that the adrenalectomized animals were less severely traumatized than the intact animals. The degree of water excretion is dependent upon the degree of trauma, and this is difficult to reproduce accurately so that comparison between various groups is not easy. For this reason it was decided to use histamine as a damaging agent, since the degree of damage can be controlled by the amount of histamine injected. In figure 5 are shown the results of the intraperitoneal injection of 15 mgm. of histamine di-hydrochloride, given at the same time as the saline. The effect of the single injection of histamine, whilst very marked in the first three hours, wore off more rapidly than that of the surgical trauma. In figure 6 are shown the results in a group of 12 adrenalectomized animals, used seven days after the adrenalectomy and maintained during this time on saline. One and five-tenths milligrams of histamine di-hydrochloride were used and it will be seen that the effect of this much smaller dose of histamine persisted longer in the adrenalectomized animals than that of the 15 mgm. dose in the intact group in figure 5, but that by 23 hours the percentage of water excretion was comparable with that in the untreated adrenalectomized group shown in figure 2. In a further group of twelve animals the effect of 2 cc. of cortin in increasing the rate of the excretion at the three and six hour periods is clearly shown. In this experiment sodium and chloride determinations were done. In the group without the cortin treatment 38 per cent of the injected Cl and 29 per cent of the injected sodium were excreted at the end of six hours and 150 per cent of the injected Cl and 90 per cent of the sodium at the end of 23 hours. In the cortin treated group 68 per cent of the injected Cl and 52 per cent of the injected Na were excreted in 6 hours, and 166 per cent of the injected Cl and 124 per cent of the injected Na in 23 hours. In figure 7 a similar experiment was performed, in which the cortin used had been inactivated by heating to 50° centigrade for half an hour in one-tenth

normal NaOH. The inactivated cortin did not increase the rate of excretion.

Since the experiments of Selye have shown that adaptation to a damaging agent can occur both in normal and adrenalectomized animals, it was thought of interest to investigate the effect of histamine on the rate of water excretion after pretreatment with this substance. Four intact animals were therefore injected daily with intraperitoneal doses of histamine di-hydrochloride increasing from 5 mgm. to 40 mgm. in seven days. These animals and a group of untreated controls, were then injected with 40 mgm. of histamine di-hydrochloride at the time of the saline injections. The results are shown in figure 8 and the rapid wearing off of the effect of histamine in the six hour period is again seen in this experiment. In fact both the pretreated and control groups excreted more water at this time than did the untreated intact animals in figure 1. At the 23 hour period the excretion in both groups was much greater (130 per cent) than in any other group of animals, as if the later effect of large doses of histamine was to increase the water excretion above that of the untreated intact animals. At three hours, however, the pretreated group showed a much greater water excretion than the non-treated group. The amount was in fact equal to that in the normal controls of figures 1 and 5. In the case of the adrenalectomized animals, the pretreatment was begun on either the second or the third day after the adrenalectomy, and the experiment was performed on the 7th day. Intraperitoneal injections of histamine dihydrochloride were gradually increased from 0.5 mgm. to 1.5 mgm. and the amount of the intraperitoneal injections at the time of the experiment was 1.5 mgm. The control group was adrenalectomized at the same time but had no pretreatment. The results are shown in figure 9 and the effect of this pretreatment in increasing the rate of excretion of water over that in the control group is clearly seen.

The effect of pretreatment with histamine on the water retaining action of surgical trauma is shown in figure 10. Four normal rats were pretreated with intraperitoneal injections of histamine, gradually increasing from 3 to 100 mgm. in ten days. Untreated rats were used as controls. At the time of the injection of saline all rats had the stomachs squeezed three times with an artery forceps. It can be seen in the figure that the histamine pretreated animals excreted the injected fluid more slowly than did the non-pretreated animals.

Discussion. The tendency under conditions of damage to retention of intravenously injected saline is clearly demonstrated in the foregoing experiments. In figure 2 the water-retaining effect of mild damage is seen to be greater in the adrenalectomized than in the intact animal. The mechanism whereby this retention of water and its transfer to extravascular spaces (as evidenced by the occurrence of edema and pleural and peri-

toneal transudates) is not determined. That the edema tendency is not due to a local effect on the kidney alone is rendered probable by the experiments of Selye (5) in which he showed that edema occurred after damage in the nephrectomized rat at a time when no edema was present in the nephrectomized controls. The mechanism may be dilatation and increased permeability of capillaries which has been put forward by Moon (6) and others as the basic feature of shock, but in the "alarm reaction" (especially after such damaging agents as muscular exercise or cold) retention of water may occur without clinical evidence of shock.

The results of the present experiments and those of Karady et al. (7) showing that various damaging agents cause retention of water given by stomach tube to rats should be borne in mind when assays of urinary or other crude extracts for the anti-diuretic principle of the posterior pituitary are done. Toxic substances present in the extracts may cause retention of water, a result which may be interpreted as indicating the presence of the specific anti-diuretic principle.

The effect of cortin in increasing the percentage of water excreted by the adrenalectomized rat after intestinal manipulation and after histamine injection is shown in figures 4 and 6. This is not surprising in view of the known action of cortin in shifting water from extra-vascular spaces into the blood stream in the adrenalectomized animal. Swingle et al. (8, 9) have shown that cortin apart from its effect in restoring blood volume also tends to raise blood pressure and to overcome the capillary atony present in damaged adrenalectomized dogs. This would favour increased urinary secretion. It may be noted that cortin increased the amount of sodium excreted by the adrenalectomized animal after injection of histamine. This is in contrast to the usual sodium—retaining effect of cortin after adrenalectomy. Swingle et al. (10) found that in the adrenalectomized dog under water intoxication cortin increased the rate of water excretion but not that of sodium. Under the conditions of our experiments sodium and water were retained in a parallel manner and the effect of cortin was the same on both and also on chloride excretion. It is possible that the use of intravenous saline rather than of water by mouth may have modified the action of cortin on sodium excretion.

Cortin had no effect on water excretion in the intact animal after intestinal manipulation (fig. 3). This absence of effect may have been due to inadequate dosage since it is known that much larger amounts of cortin are required to produce effects of any kind in the intact animal.

The use of larger doses was prevented partly by the presence of 0.1 per cent tricresol in the cortical extract. This was found to be slightly toxic to the animal when large volumes of extract were given. Originally in view of the hypertrophy of the adrenal cortex which was shown by Selye (1) to occur during the alarm reaction at about the same time that the edema

disappeared it was thought that cortin given early might hasten the disappearance of the edema even in the intact animal. Recent observations by Weil and Browne (11) indicate that there is an excretion of cortin in the urine of patients after surgical operations within 24 hours of the operation, indicating possibly that there is an increased secretory activity by the adrenal cortex occurring very rapidly after damage.

The demonstration that damage causes sodium retention and that cortin promotes excretion of sodium under these conditions is of interest in connection with the assumption that a substance which causes sodium retention is cortin-like in other respects. Thorn and Harrop (12) and Thorn and Engel (13) have shown that oestrogens and other sex hormones cause sodium and chloride retention in normal and adrenalectomized dogs and in human beings, cortical extracts and corticosterone have a similar effect. The similarity between oestrogens and cortin ceases when one considers the effect of the former in decreasing the time of survival of the adrenalectomized rat as shown by Selye et al. (14), Schacher et al. (15) and Cavanaugh and Gaunt (16). Schacher et al. (15) also found testosterone to be toxic. Progesterone on the other hand was found by Gaunt and Nelson (17) to prolong the life of the adrenalectomized rat. Thorn and Engel (13) found that the sex sterols did not increase the survival of the adrenalectomized dog. These results show that the production of sodium retention by a sterol does not necessarily indicate that its action will be cortin-like in other respects. The last group of experiments on the effect of pretreatment with histamine requires little comment. They demonstrate that a degree of adaptation to this substance can occur after pretreatment even if the adrenal is not present during the period of pretreatment. The last experiment (fig. 10) shows that pretreatment with histamine does not alter the action of intestinal manipulation in causing water retention. This is of interest because of the demonstration by Karady (18) in the rabbit that pretreatment with histamine reverses the effect of intestinal manipulation on blood volume and prevents the development of shock.

SUMMARY

The effect of gastric and intestinal manipulation and of histamine injection on the urinary excretion of intravenously injected saline has been studied in normal and adrenalectomized rats. It has been found that there is marked water retention and edema formation under the influence of these damaging agents. The degree of water retention parallels the intensity of the damage. The doses of cortin used have no effect on the excretion of water after surgical trauma in the intact animal nor does cortin affect the rate of water excretion in adrenalectomized animals otherwise undamaged. Cortin markedly increases the rate of water excretion

in adrenalectomized animals after surgical trauma or injection of histamine. It also increases the rate of sodium and chloride excretion under the conditions of the experiment. After pretreatment with histamine the effect of histamine in producing retention of injected saline is lessened in both intact and adrenalectomized animals, even if the pretreatment is begun after adrenalectomy.

The effect of damaging agents in producing water and sodium retention should be borne in mind in assaying crude extracts for anti-diuretic activity and in drawing conclusions as to the cortin-like nature of the action of certain compounds in causing sodium retention.

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BLOOD PRESSURE VALUES IN STREET DOGS

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The study of experimental hypertension, which is concerning so many physiologists at the present time, justifies an attempt to outline statistically the blood pressure findings in the canine population which comes into the laboratory. The study which we present here is purely descriptive and relates itself to the following points: 1, the method and its accuracy; 2, the relation of pressures in the femoral artery to those in the arteries of the anterior part of the body; 3, the use of morphine to secure a tranquil state for the determination; 4, the laborious use of training to secure a similar tranquil state during arterial puncture; 5, the mean pressures of the series and a statistical survey of its variation in the population studied; 6, the relation of the variation to sex and age and the season of the year in which observations were made; 7, pathological findings in a sample of the dogs, and 8, the effect of barbiturate narcosis, thiocyanate therapy and sympathectomy in dogs with high blood pressure.

Blood pressures were recorded optically by means of the hypodermic manometer (1, 2). The records were measured for systolic, diastolic and dicrotic pressure by means of calibration records made with a mercury manometer and the measurements are accurate to within 4 or 5 mm. Hg.

In interpreting the figures it should be realized that the pressures reported here are from the femoral artery and that systolic pressures from this part of the body are known to be about 40 mm. higher than simultaneous pressures taken from the aortic arch (3). With this in mind there is no reason why one should expect that pressures taken from the carotid should agree with pressures taken from the femoral (4). Thus the mean systolic pressure of our 215 dogs was 179.5 mm. Hg and their diastolic pressure averaged 88.6 mm. Hg. Subtracting 40 mm. from the systolic pressure would indicate that the average pressure in the carotid arteries of our dogs is 139/88, a figure which is not significantly different from that reported by Gregg et al. for carotid pressures in trained dogs. The areas of the femoral and aortic pulses are nearly the same and thus the mean pressures are within a few millimeters of the same figure.

The dogs in our series were not trained. The experiments were performed in the afternoon. The dogs had been fed the night before and had been given 3 mgm. per kgm. morphine 20 to 60 minutes before the punctures were made. They were somnolent and completely amenable to the experiment.

In order to see what the effect of training might be, however, two dogs, not included in the above series, were trained to lie on the table while the punctures were being made. It was possible to train these dogs so that their pressures were similar to the values initially under morphine. The trained dogs showed no sign of excitement or apprehension during the procedure, had normal heart rates and had pressure 50 to 60 mm. less than early in the training. Nevertheless the trained dogs never showed pressures that were significantly lower than the initial observations under morphine.

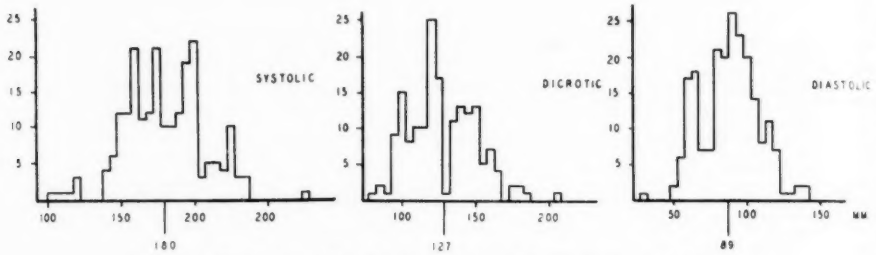

Fig. 1. Frequency distribution of arterial pressure in 215 dogs

We feel that the figures reported from the morphinized dogs are perhaps a little below what the animal supports when he is undertaking an average day's routine and a little above what they would be when the animal is asleep.

In interpreting blood pressure data from dogs one must recognize that there is a wide biological variation in animals which have all been treated alike. The chart (fig. 1) shows the distribution of arterial pressures among 215 dogs. The systolic pressure ranges from 100 to 275 with a mean of 180. The diastolic ranged from 30 to 140 with a mean at 89. The dicrotic pressure, which seems on good evidence to be 15 or 20 mm. less than the systolic pressure in the fore quarters, ranges from 85 to 205 with a mean at 127.

Sex. Ninety-nine males and 116 females had systolic, diastolic and dicrotic pressures which were not significantly different either from the mean of the whole group or from each other.

Age. While it is impossible to gauge the age of a dog with any accuracy from examination of the teeth, it is possible to separate the very young

dogs with sharp, white, immature teeth from the rest of the group. Twenty-two dogs in this classification had pressures of 166/77 (119 dicrotic). We were unable to find any further correlation between blood pressure values and apparent age.

Season of the year. Early in our work we gained the impression that pressures were higher in the winter than in the warm months of spring and fall. This impression was verified by an analysis of our figures by months as shown in table 1.

The individual dogs which we trained showed significantly lower blood pressures in hot summer weather and higher pressures over the winter, and a group of dogs with unusually high blood pressure showed marked reduction in the summer. All of these dogs which were under continuous observation remained in good health and spirits throughout the whole time they were watched.

TABLE 1

Average monthly blood pressures in dogs

MONTH	DOGS	SYSTOLIC PRESSURE	DIASTOLIC PRESSURE	DICROTIC PRESSURE
January	26	187	96	134
February	20	208	104	146
March	19	186	90	131
April	26	173	88	120
May	27	176	80	113
October	35	167	79	125
November	48	184	89	128
December	11	184	93	134

Pathological findings. Sections from heart, liver, pancreas, kidney and sometimes the intercostal muscles were studied from 55 dogs. A well developed muscular coat of the small arteries and arterioles was very commonly encountered. In our earlier report (5) this was confused with a hypertrophic sclerosis, but its frequent occurrence and the absence of concurrent pathological or physiological findings leads us to regard this as a normal condition in dogs' tissues. This series was representative of the whole series in that the distribution of pressures in the two series was similar.

Hyalinization of an occasional arteriole was observed in six dogs. Five of these dogs had pressures above the average of the series (214/123, 185/106, 225/110, 220/105, 226/124). The sixth dog was to all appearances a very old animal with the highest heart-weight-body-weight ratio of the whole series and some of the glomeruli showed fibrosis. It is possible to regard this animal as one whose heart had suffered senile deterioration in the face of long continued hypertension.

When the dogs were grouped in a series so that the heart-weight-body-weight ratio increased, there was found to be a concomitant increase in the average blood pressure. This was small in amount and of doubtful statistical significance.

Glomerular nephritis was observed in two of the dogs, acute glomerular nephritis with blood pressure of 200/104 and without arteriolar damage; second stage glomerular nephritis with blood pressure of 226/124, associated with occasional hyalinized arterioles. In no instance was nephrosclerosis observed though one dog (185/106) had hyalinized arterioles, ischemic atrophy of some glomeruli and some cortical scarring.

Effect of barbiturate anesthesia. Five dogs with highest pressures were subjected to several experimental procedures with the hope of working out the mechanism which is responsible for their hypertension. They were first given barbiturate anesthesia of surgical depth (Neonal or Nembutal). Pressures which had averaged 222/101 fell to 137/67. The individual animals showed very similar reductions in pressure.

Effect of sodium thiocyanate. This drug was injected in these five hypertensive dogs in such quantities as to keep the plasma concentration between 10 and 20 mgm. per 100 cc. as determined by the method of Crandall and Anderson (6) for a month's time. The dosage was then increased so as to give a concentration between 30 and 50 mgm. per 100 cc. and pressures were observed for another month. Observations were continued during the period of excretion and for two months afterward. The rate of excretion in different animals varied over a threefold range. There was a definite reduction in blood pressure during the observation period which persisted for two months after the blood had become free of thiocyanate. The pressure fall was of the same order as that attributed above to the season of the year, and as summer was coming on we do not believe that the drug had anything to do with it.

Effect of sympathectomy. The operation was performed on five hypertensive dogs which had showed lowered pressures under barbiturate anesthesia. The splanchnic nerves were cut just above the diaphragm and several segments of the sympathetic chain were removed. The operation was performed under artificial respiration as described elsewhere (7) and was found at autopsy to be apparently successful in four dogs in severing autonomic connection between the thoracic segments and the viscera. Regeneration had been prevented by the use of silver clips on the central stumps.

The operation caused a fall of pressure lasting in all cases less than two weeks. After this time the pressure rose to the hypertensive level previously observed.

Discussion. The wide range in dogs' arterial pressure and the seasonal changes are factors which should be kept in mind by those interested in

experiments designed to change dogs' blood pressure. The extremely muscular coats in dogs' arterioles is no doubt a reflection of an extremely active vasomotor life. Any one who has seen a dog sleeping quietly one moment and then quivering with frantic excitement the next must marvel at the vascular machinery that makes such activity possible. Indeed the large hearts which dogs have as compared to many other animals would be incompatible with anything but a very sturdy vascular tree.

The comparatively low summer blood pressures are probably related to cutaneous flushing for heat dissipation. A dog's skin feels hot in warm weather in contrast to the moist coolness of perspiring human skin.

The ineffectiveness of sympathectomy as compared with the results of barbiturate anesthesia may be related to the restricted field which was denervated, to the development of myogenic tone in the denervated parts as well as to the fact that barbiturates produce a muscular as well as a nervous depression. All evidence indicates that there is rarely any "arteriolar fixation."

This investigation was made possible by a grant from the American Medical Association.

SUMMARY

Optical records were made of the arterial pressures of 215 street dogs under light doses of morphine. The pressures were found to vary widely in dogs that had all been treated alike.

Very young dogs have pressures lower than the average. Dogs have lower pressures in the warm months than in the cold months. Dogs with hyalinized arterioles and with glomerular nephritis have higher pressures than the average population.

Neither thiocyanate nor sympathectomy produced a significant permanent lowering of blood pressure in hypertensive dogs whose pressures fell under barbiturate narcosis.

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DIRECT ARTERIAL AND VENOUS PRESSURE MEASUREMENTS IN MAN AS AFFECTED BY ANESTHESIA, OPERATION AND SHOCK[1]

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Anesthesia and surgical operations as commonly performed in man are an ideal experimental set-up for study of human blood pressure changes. By means of the hypodermic manometer (1, 2) we have obtained nearly continuous tracings of the arterial blood pressure throughout surgical procedures lasting several hours. From a study of the records we can describe: 1, the differences in the normal pressure pulse contour shown by various individuals; 2, the changes which befall these contours during anesthesia, operation and the withdrawal of anesthesia; 3, the changes which occur during the development of "shock" and when the arterial pressure gets so low that the anesthetist warns the surgeon that he "can not get the pressure." Some of our arterial records are accompanied by calibrated recordings of the A, C and V waves of the venous pulse, presented here so far as we are aware for the first time from man.

The approach which we have made is mostly descriptive. We cannot as yet commit ourselves very definitely as to the theoretical meaning of the phenomena we are describing. We are certain, however that the curves do actually represent the true pressure pulses in the vascular tree.

In figure A 1–4 appear pre-anesthetic curves from four individuals who have been pre-medicated with morphine sulfate and scopolamine hydrobromide about one hour before. Although the systolic and diastolic pressures are within normal limits, it will be noticed that there are definite qualitative differences in the shape of the curves. The first curve (A–1) with its high, narrow, initial peak, marked secondary wave and flat diastolic portion we have come to call a pulse of the empty type. When we compare the pressures as directly measured from this curve with those obtained by the auscultatory method we find that the latter usually gives systolic values too low, diastolic values nearly correct or somewhat too high. The high narrow pressure wave cannot effectively pass under the

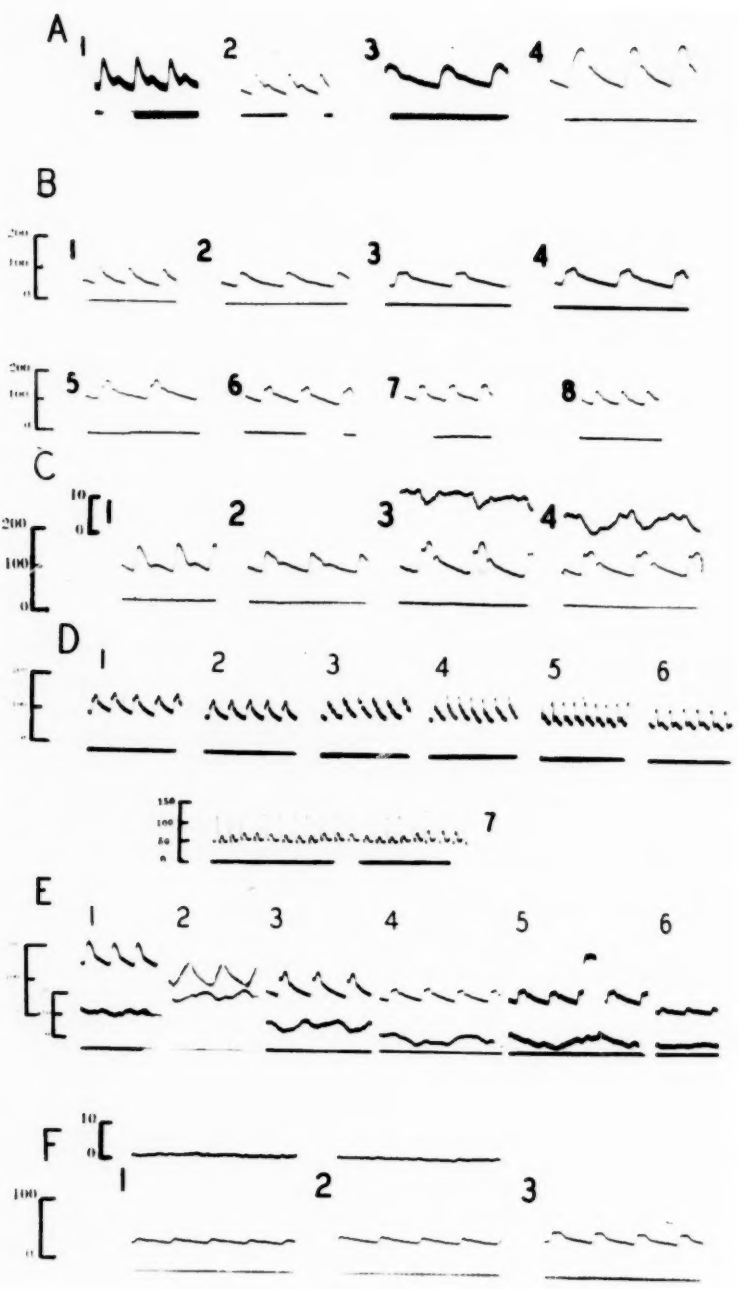
[1] This work was reported in part before The Section on Miscellaneous Topics, Session on Anesthesia, of the American Medical Association May 17, 1939.

inflated cuff until the pressure therein is definitely below that registered at the peak of the curve. The second curve (A–2) is one in which the peak is not so narrow and the diastolic portion not so flat. We think of such a curve as a moderately empty pulse. The third (A–3) is a moderately full pulse, one whose peak is rounded and wide, which lacks the secondary wave and whose diastolic portion is sloping. The fourth (A–4) is a full pulse. It has a well maintained systolic portion and a steeply sloping diastolic portion. When auscultatory pressures are taken from individuals showing this type of contour the agreement with the direct determination of the systolic pressure is better than when the contour is of the more empty type.

In figure B 1–8 there is a series of arterial pulse contours taken from a young colored male who was given cyclopropane anesthesia for amputation of a leg. The pertinent physical findings were a positive blood Wassermann and dry gangrene of the left foot. The anesthesia was uneventful. Systolic and diastolic pressures rose steadily during the procedure from an initial 104/60 to 155/110 and after the anesthesia was discontinued the pressure fell back to 129/93. The contours changed from a moderately empty type to the moderately full type and then back. More or less parallel with the change in fullness of the contours was a slowing of the heart, offering more time for the diastolic filling and consequent increase in stroke volume of the heart.

Figure C 1–4 are contours taken from a middle aged colored female who was given cyclopropane anesthesia for a total abdominal hysterectomy. Her physical examination revealed a moderate secondary anemia and a large fibroid uterus. Contour C–1 is before anesthesia. This is an empty type of pulse, both pressure and pulse rate are higher than normal, indicating some apprehension. C–2 was taken during deep first plane anesthesia (3), note the change to a fuller contour, the systolic pressure has dropped slightly, the pulse rate is about the same as before. Contour C 3 was taken during upper second plane anesthesia. The arterial contour is now fuller and the pressure is much higher although the course of the anesthesia was apparently smooth. The venous contour distinctly shows the A, C, V complex and the pressure is much higher than normal. Pulse C–4 was taken during deep second plane anesthesia. Both the arterial and venous pressures have fallen. The arterial pulse is still full but has assumed more of a plateau form. The venous contour shows a pressure rise throughout diastole which indicates a slower filling of the right ventricle.

Figure D 1–6 are contours obtained from a young negro female, age twenty-nine, who was given cyclopropane anesthesia for a total abdominal hysterectomy. Her physical examination was not remarkable except for a large uterine fibroid and chronic bilateral salpingitis. The induction



Figs. A–F

of the anesthesia and the subsequent two-thirds of the procedure appeared uneventful (D 1, 2). Towards the end of the operation the pulse rate increased and the contour became progressively more empty (D 3, 4, 5). The discontinuance of the anesthesia when the operation was completed was followed by a more marked increase in pulse rate and a moderate reduction in arterial pressure (D 6). This was a particularly difficult hysterectomy with a more than usual degree of bleeding and trauma. The patient's skin was cold and moist, the mucous membranes were pale. Her apparent circulatory volume deficiency was corrected with the administration of hypertonic glucose and a blood transfusion.

In figure D-7 are axillary arterial contours taken about one-half hour after discontinuance of cyclopropane anesthesia for an exporatory laparotomy. The patient was a colored female of thirty-seven years of age. Her pertinent physical findings were a secondary anemia (11 grams of Hb), an essential hypertension (195/95) and a large intra-abdominal tumor arising from the pelvis which was suspected of being a fibroid uterus with malignant changes (sarcoma?). The induction of the anesthesia was

Fig. A 1-4. Pre-anesthetic pulses: A-1 empty type pulse, B. P. 132/76, H. R. 96; A-2 moderately empty pulse, B. P. 100/55, H. R. 60; A-3 moderately full pulse, B. P. 120/62, H. R. 60; A-4 full pulse, B. P. 170/85, H. R. 72.

Fig. B 1-8. Pressure pulses during cyclopropane anesthesia for amputation of leg. B-1 before anesthesia, H. R. 84; B-2 induction of anesthesia, H. R. 64; B-3 deep first plane, H. R. 64; B-4 light second plane, H. R. 64; B-5 deep second plane, H. R. 78; B-6 light second plane, H. R. 84; B-7 light first plane, H. R. 104; B-8 recovery after anesthesia discontinued, H. R. 120.

Fig. C 1-4. Pressure pulses during cyclopropane anesthesia for total abdominal hysterectomy. C-1 before anesthesia, H. R. 100; C-2 deep first plane, H. R. 90; C-3 upper second plane, H. R. 76 (upper curve venous, lower curve arterial); C-4 deep second plane, H. R. 76.

Fig. D 1-6. Pulse contours during cyclopropane anesthesia for total abdominal hysterectomy. D-1 third plane anesthesia one hour after induction, H. R. 80; D-2 deep third plane, H. R. 90; D-3 return to second plane, H. R. 110; D-4 return to first plane, H. R. 125; D-5 just before anesthesia is discontinued, H. R. 144; D-6 anesthesia discontinued, H. R. 144.

Fig. D 7. Axillary pulse contour one and a half hours after exploratory laparotomy under cyclopropane anesthesia in which hemorrhage and severe trauma occurred, H. R. 140.

Fig. E 1-6. Pressure pulses during cyclopropane anesthesia for total abdominal hysterectomy. Upper curve arterial lower curve venous. E-1 before anesthesia, H. R. 110; E-2 second plane, H. R. 70; E-3 mid-second plane, H. R. 70; E-4 radial pulse curve late in anesthesia low first plane, H. R. 68; E-5 brachial pulse contour low first plane, H. R. 74; E-6 anesthesia discontinued, H. R. 84.

Fig. F 1-3. Pressure pulses one hour after discontinuance of cyclopropane anesthesia for radical amputation of a breast. Upper curve venous, lower curve arterial. F-1 before intravenous infusion; F-2 after 150 cc. of 5 per cent glucose intravenously; F-3 after 350 cc. of 5 per cent glucose intravenously. Pulse rates, F1, 104, F2, 104, F3, 90.

smooth. Exploration of the abdomen and an attempt to remove the tumor which was extremely vascular and bound down by numerous fibrous adhesions had to be abandoned. The patient's condition became extremely grave due to hemorrhage and trauma. During the operation, one hour and a quarter after induction of anesthesia, the pulse began to rise and the blood pressure to fall. The patient started to perspire profusely and soon the skin became cold and clammy. In fifteen minutes the circulation which had originally stabilized at a blood pressure of 140/80 and pulse 80 began to progressively fail. The arterial pressure dropped to 60/40 and the pulse rate increased to 110. Five hundred cubic centimeters of 10 per cent glucose were given intravenously with little change in the patient's condition. More fluids (5 per cent glucose) were given and after about one hour of this type of therapy the blood pressure rose to about 105/70 pulse rate 140. It was impossible to find a good blood match for transfusion. She died in "shock" two and a half hours following operation.

At the time the records were taken (D 7) the patient presented an even more alarming appearance than the patient of figure D 1–6. These two patients are in marked contrast to patients E and F whose actual blood pressures were lower but whose clinical appearance never gave us occasion for alarm. Contours D 5, 6 and 7 are typical of those we associate with oligemia. The very rapid empty pulses are to be associated with a more serious condition than the slow full pulses to be considered next though the latter may show pressures decidedly lower.

Figure E 1–6 depicts contours of arterial and venous pressures taken from a colored female of middle age during cyclopropane anesthesia for a total abdominal hysterectomy. The only physical abnormalities of this patient were a moderate secondary anemia and uterine fibroids. What appeared as adequate pre-medication (morphine gr. 1/6 and scopolamine gr. 1/150 given one and a half hours before) and an apparent calm exterior were belied by the pre-anesthetic contour (E–1) which shows an extremely high arterial pressure 203/146 (normal pressure 124/80), an accompanying high venous pressure (13 mm. Hg) and a tachycardia of 120. The induction of the anesthesia, nevertheless, was quite uneventful. The patient was then placed in a twenty degree Trendlenburg position and anesthesia was deepened to low second plane. Tugging on the uterus shortly after the usual abdominal packing for pelvic surgery produced a laryngeal stridor of several minutes' duration (E–2). The latter or the change in position may account for the abnormally high venous pressure (22 mm. Hg) and the still elevated arterial pressure. The anesthesia and the surgical procedure then progressed smoothly (E–3). The arterial (127/56) and the venous (6 mm. Hg) pressures fell to within normal limits. At the end of one hour, following the closure of the peritoneum, the

anesthesia was gradually brought to a lighter plane by washing out part of the anesthetic mixture with air and oxygen. A fall in both arterial and venous pressures ensued (E–4, A.P. = 82/48; V.P. = 3; E 5 A.P. = 89/50; V.P. = 2). This fall in pressures progressed with the complete discontinuance of the anesthesia when the surgery was completed (E 6 A.P. = 65/47; V.P. = −1). This drop in arterial pressure occurred in a number of cases of our series when cyclopropane anesthesia was discontinued. Clinically the patient appeared warm and dry in spite of being somewhat pale and the mucous membranes sub-cyanotic. The pulse contour E–6 although showing a very small pulse pressure is different from the low pressure contour (D–6 and 7) associated in our minds with hemorrhage or traumatic shock.

Figure F 1–3 is an example of the same type of contour as E 6. These tracings were taken one hour after discontinuance of cyclopropane anesthesia in a middle aged white female who had undergone a radical amputation of a breast. This case differs somewhat from that previously described in that three quarters of an hour after anesthesia and operation had begun, suddenly the arterial pressure fell and the pulse became slower. Clinically the patient did not present an alarming appearance, she remained warm and dry. Nevertheless 500 cc. of 10 per cent glucose were given intravenously and the pressure slowly rose to its original normal level. A second drop in arterial pressure occurred shortly after this patient was returned to bed. This time the individual was conscious but uncooperative. Apomorphine gr. 1/40 was given intramuscularly and within eight minutes she became quiet and fell asleep. Contour F–1 was then taken (A.P. = 43/29, V.P. = 1). An intravenous infusion of 5 per cent glucose was started. Contour F–2 shows the effect of 150 cc. of this fluid (A.P. = 58/41, V.P. = 1). Contour F–3 is after 350 cc. of fluid had been given (A.P. = 98/54). Note the full contour of this pulse curve. The patient was given a total of 500 cc. of the above infusion, her blood pressure remained elevated and she made an uneventful recovery.

It may be difficult to determine the auscultatory pressure values in pulses of the type shown in E–6 and F–1. Unless pressure in the cuff is lowered very slowly the Korotkoff sounds are either not heard or sound very vague. Even with greatest care large irregular errors are made in determining the pressure.

During abdominal surgery various kinds of manipulations (4) produced a lowering of the systolic and diastolic pressures accompanied not infrequently by a slowing of the heart (fig. G 1–9). G–2 shows the effect of raising the gall bladder rest; G–4 the result of lowering the rest; G–6 the effect of tying off the gall bladder; G–5 the result of inserting the hand into the upper abdomen; and G–8 the effect of pressure over the coeliac plexus. Contours G–1, 3, 7 and 9 show the type of change which actually

occurs in the pulse curve. The contours show a drop in both systolic and diastolic pressures, the former more than the latter, with a narrowing of the pulse pressure. However, the pulse remains full and differs from the type of contour we associate with pressure falls in oligemia or traumatic shock. These neurogenic drops in pressure occurred immediately after the above manipulations. Recovery was not always prompt and not in all cases complete. The accumulation of the results of such manipulations may account for the fall in pressure sometimes seen in patients not manifesting secondary traumatic shock (e.g., E–6, F–1). Intensification of the after effects of these stimuli may well be the explanation for the dramatic

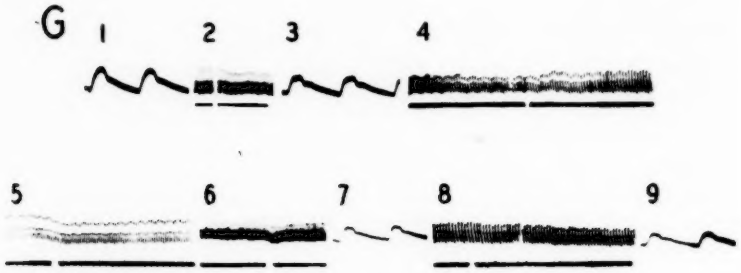


Fig. G 1–9. Pressure pulses during cyclopropane-ether anesthesia for cholecystectomies. G 1–4 effect of changing the position of the gall bladder rest; G–1 rest down B. P. 160/95, pulse rate 66; G–3 same patient gall bladder rest up B. P. 140/92, pulse rate 60; G–2 at signal gallbladder rest was raised; G–4 rest up B. P. 115/80 pulse rate 70, at signal rest was lowered B. P. 131/80, pulse rate 70. G 5 to 9, effect of surgical manipulations (gall bladder rest is up during these procedures). G–5 B. P. 137/84, pulse 84, at signal surgeon's hand explored the upper abdomen B. P. 113/75 pulse 70; G–6 B. P. 127/80 pulse 72, at signal the gall bladder was "tied off" B. P. 113/70, pulse 68; G–7 B. P. 125/80, pulse 60; G–9 same patient pressure being exerted on the coeliac plexus B. P. 102/68, pulse 60; G–8 B. P. 126/84, pulse 66, at signal pressure on coeliac plexus was started B. P. 110/70, pulse 66.

fall in arterial pressure sometimes seen when an agent like cyclopropane is suddenly withdrawn.

SUMMARY

A series of about twenty cases was followed during surgery and inhalation anesthesia. Venous and arterial pulses were optically recorded with the hypodermic manometer. Changes in the systolic and diastolic pressures as directly recorded were compared with auscultatory blood pressures as ordinarily taken by the anesthetist. It was also possible to show the gradual changes in the contour of the arterial and venous pulses.

The following tentative notions have developed: 1. Both venous and

arterial contours show striking changes during surgical operation and anesthesia. 2. These changes are of two types; those presented by cases of hemorrhage or secondary traumatic shock with rapid pulse of the "empty" type, and those presented by shock of neurogenic origin with the equally low pressures, slow heart and pulse contours of an entirely different shape.

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THE CARDIO-ACCELERATOR FIBERS IN THE VAGUS NERVE OF THE DOG

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For many years, physiologists have disagreed about the presence of cardio-accelerator fibers in the vagus nerve. A number of investigators, including Rutherford (1869), Schiff (1878), and Tulgan (1923) observed an increase in heart rate on electrical stimulation of the peripheral end of the cut cervical vagus after paralysis of the cardio-inhibitor endings with atropine. On the other hand, Boehm (1875), Rossbach (1875), and more recently Hering (1924) vigorously denied that stimulation of the vagus nerve after atropine could accelerate the heart.

Opinion was thus divided when Jourdan and Nowak (1934 and 1936) presented rather conclusive evidence for the existence of true vagal cardio-accelerators in the dog. They were able to demonstrate acceleration of the heart on electrical stimulation of the peripheral end of the cut vagus rootlets intracranially after atropine. These investigators performed cross-circulation experiments in which the isolated head, connected to its trunk only by the vagus nerves, was perfused by a donor animal. Acute anemia of the isolated head resulted in acceleration of the atropinized heart; the effect disappeared when the vagus rootlets were sectioned.

The significance of these vagal accelerators has been indicated by Brouha, Cannon, and Dill (1936) who found, in sympathectomized dogs, that exercise or emotion could increase the pulse rate well above the automatic rhythm of the completely denervated heart. This observation has been confirmed in dogs by Brouha, Dill, and Nowak (1937), and in cats by Hodes (1939). Reflex activation of the cardio-accelerator fibers in the vagus nerve by stimulation of the central end of the superior laryngeal nerve in the dog has been reported by Jourdan and Morin (1936).

The present experiments were undertaken in an attempt to confirm and perhaps extend the observations of Jourdan and Nowak (1936) on the vagal accelerators. Adult dogs were anesthetized with chloralose injected intravenously in doses of 65 mgm. per kilo of body weight. Blood pressure and heart rate were recorded from the carotid artery by means of a mercury manometer on a smoked kymograph drum. In some experiments, the Hurthle manometer was employed to record heart rate.

The Harvard inductorium was used to provide electrical stimulation of moderate current strength. Atropine sulphate was given intravenously in doses of 0.1 mgm. per kilo of body weight.

I. *Effects of electrical stimulation of the cervical vago-sympathetic trunk after atropine.* The intact vago-sympathetic nerve was stimulated after atropine at various levels in its course through the neck, reflex effects through the sympathetic nerves or reflex secretion of adrenalin having been eliminated by transection of the spinal cord at the second cervical level. Artificial respiration was maintained by a pump connected with the

TABLE 1

Effects of stimulation of vagus nerves at various levels

POINT OF STIMULATION	SYMPATHETIC NERVES INTACT				UPPER THORACIC SYMPATHECTOMY			
	Number of experiments	Number that showed acceleration	Average acceleration	Maximal acceleration	Numberof experiments	Number that showed acceleration	Average acceleration	Maximal acceleration
I. Right vago-sympathetic nerve								
a. At level of hyoid bone	7	4	11	20	9	7	30	66
b. At lower end of larynx	15	13	28	56	6	6	37	60
c. At inferior cervical ganglion	12	12	75	104	2	2	52	88
II. Left vago-sympathetic nerve								
a. At level of hyoid bone	6	1	4	8				
b. At lower end of larynx	14	2	3	10	8	5	13	18
c. At inferior cervical ganglion	9	7	42	96	2	2	22	24
III. Right vagus rootlets	6	3	21	32	1	1	24	24
IV. Acute cephalic anemia	8	5	20	36	17	15	32	66

tracheal cannula. In some experiments, the peripheral end of the cut nerve was also stimulated.

In eighteen experiments the nerves were stimulated with the sympathetic cardio-accelerator innervation intact. The results of stimulation were very different at different levels in the course of the same nerve through the cervical region, as shown in table 1. When the right vago-sympathetic trunk was stimulated at about the level of the hyoid bone, the acceleration was only very slight, and in some dogs was entirely absent even when a strong stimulus was used. The acceleration was more

marked the closer the point of stimulation to the inferior cervical sympathetic ganglion, and the right vago-sympathetic nerve was much more effective than the left. In all cases, acceleration occurred after a relatively long latent period and the effect persisted for some time after cessation of stimulation, characteristic of the sympathetic influence on the cardiac rhythm. There was little or no change in blood pressure associated with the accelerator response of the heart. Stimulation of the left vago-sympathetic nerve only resulted in cardiac acceleration when the electrodes were placed on the inferior cervical ganglion.

In a further series of eleven experiments, stimulation of the vago-sympathetic nerves was preceded by bilateral upper thoracic sympathectomy, as well as by section of the spinal cord at the second cervical level (table 1). Excitation of the right nerve high in its cervical course with a moderate stimulus was much more effective than before sympathectomy, producing an average acceleration of 30 beats per minute. Stimulation of the left vago-sympathetic nerve in its mid-cervical portion resulted only in slight acceleration or none at all, the average increase in heart rate amounting to 13 beats per minute. It is thus evident that the cardio-accelerator fibers predominate in the right vagus nerve.

There is reason to believe that the increase in heart rate obtained during stimulation of the upper right cervical vago-sympathetic trunk is not the result of stimulation of the superior cervical ganglion or its post-ganglionic fibers. It has been impossible to produce a change in heart rate by stimulation of the right superior cervical sympathetic ganglion, although in the same dog stimulation of the right vagus nerve in this region readily evoked cardiac acceleration. This is in agreement with the results of Hering (1924), who found no change in heart rate on excitation of the cervical sympathetic above the inferior cervical ganglion in dogs, cats, rabbits, and monkeys. In man, according to Kuntz (1934), the superior and middle cardiac nerves have no effect on the cardiac rhythm.

II. *Stimulation of the vagus rootlets intracranially.* In order to demonstrate the origin of the accelerator fibers of the vagus nerve, the right vagus and accessory rootlets were stimulated intracranially at their junction with the medulla oblongata in six dogs after administration of atropine. The intact rootlets were stimulated with an insulated hook electrode, reflex effects through the sympathetic system having been eliminated by transection of the spinal cord at the second cervical level. The results of stimulation are recorded in table 1. In three experiments, moderate stimulation of the rootlets after atropine resulted in an increase in heart rate. In the other three experiments, although the basic heart rate averaged 150 beats per minute, and although the rootlets were irritable as demonstrated by marked cardiac inhibition on stimulation before atropine and by contraction of neck muscles due to spread of the current to the

spinal accessory nerve, it was impossible to change the heart rate by excitation of the rootlets. In the same animals, stimulation of the right vago-sympathetic nerve in its mid-cervical portion resulted in cardiac acceleration. In these experiments, positive results are probably more significant than negative ones. The vagal cardio-accelerator fibers thus originate with other vagus fibers in the medulla oblongata, as Jourdan and Nowak (1936) stated in their report.

Jourdan and Nowak (1936) also reported that electrical stimulation of the vagus rootlets even without atropine gave rise to cardiac acceleration. This procedure was repeated in eight dogs and their observation could not be confirmed, marked inhibition of the heart resulting from stimulation of either the bulbar accessory or vagus rootlets, even though the greatest care was taken to avoid spread of current. Excitation of the uppermost vagus rootlets, which contain only afferent fibers, had no effect on the heart rate. Furthermore, other investigators who made careful studies on the effects of stimulation of the vagus and accessory rootlets intracranially, without atropine, such as Grossmann (1895), working with rabbits and Kreidl (1897) working with monkeys, never observed cardiac acceleration.

Attempts to activate the vagal accelerator neurons reflexly. After demonstrating that fibers of the right vagus nerve can increase the heart rate, an attempt was made to excite these fibers reflexly by stimulation of afferent fibers in the left vagus nerve. The dog was prepared for the experiment by transection of the spinal cord at the second cervical level and administration of atropine. In some experiments, an upper thoracic sympathectomy was also performed. In 17 dogs, the upper cervical portion of the intact left vago-sympathetic nerve or the central end of the cut left vago-sympathetic nerve was stimulated with entirely negative results. No change in heart rate was ever observed from stimulation of the vagus afferents, although in the same experiments acceleration was observed on direct stimulation of the motor fibers in the right vago-sympathetic nerve.

Jourdan and Morin (1936) reported that the accelerator fibers in the vagus nerve could be excited reflexly by stimulation of the central end of the superior laryngeal nerve after atropine. Their evidence that the increase in heart rate was the result of activation of the vagal accelerator fibers was based on the fact that the reflex acceleration could be elicited after bilateral stellectomy and adrenalectomy and in some experiments after destruction of the thoracic, lumbar, and sacral spinal cord. Our experiments lend no support to their claims. In six dogs, the central end of the superior laryngeal nerve was stimulated. In order to rule out any other cardio-accelerator influence except the vagus, the spinal cord was transected at the second cervical level and an upper thoracic sympathectomy was performed. First, the nerve was stimulated without atropine, resulting in reflex slowing of the heart. Then atropine was injected intra-

venously in a dose of 0.1 mgm. per kilogram of body weight. No change in heart rate was ever observed as a result of stimulation of the superior laryngeal nerve after atropine. In all of these experiments, the vagal accelerators could be activated readily by direct stimulation or by acute anemia of the brain. It seems likely that the reflex acceleration observed by Jourdan and Morin (1936) may have been due to excitation of other accelerators than those in the vagus nerve. Certainly, in their experiments in dogs after stellectomy and adrenalectomy, a reflex increase in heart rate could still occur through the upper thoracic sympathetic ganglia or by vascular transport of sympathin to the heart from sympathetic nerve endings elsewhere in the body.

Thus stimulation of the afferent fibers in the vagus nerve and in the superior laryngeal nerve fails to excite the vagal cardio-accelerator mechanism. In other experiments under similar conditions in 20 dogs, a decrease in pressure in the carotid sinus failed to change the heart rate, although acceleration resulted from more prolonged compression of the cephalic arteries, due to acute anemia of the brain. Furthermore, in chronic experiments with sympathectomized dogs, Brouha, Dill, and Nowak (1937) reported absence of cardiac acceleration on stimulation of the carotid sinus as well as on electrical stimulation of the central ends of somatic nerves. In the same animals, on the other hand, these authors noted significant increases in heart rate resulting from exercise and emotional excitement, presumably from activation of vagal accelerator neurons. These observations indicate that stimulation of most afferent nerves fails to produce reflex acceleration through the vagal accelerator mechanism.

Acute anemia of the brain. Direct excitation of the brain centers by acute anemia is effective in activating the vagal cardio-accelerator neurons. This was first demonstrated by Jourdan and Nowak (1936), who observed an increase in heart rate as a result of acute anemia of the isolated cross-circulated head connected to its trunk only by the vagus nerves. The findings of these authors on the effects of cephalic anemia are in complete agreement with those to be reported here.

A total of 25 experiments was performed using acute anemia of the brain to excite the vagal accelerator mechanism. The results are shown in table 1. In two experiments the head of the experimental animal was isolated from its trunk and perfused by a donor heart-lung preparation. A heart-lung preparation was then made of the trunk of the experimental animal. The perfused head was connected to its heart only by the vagus nerves. Before administration of atropine, anemia of the head for 80 seconds was effective in greatly decreasing the heart rate. After atropine was injected into the experimental heart-lung circulation, acute anemia of the head of 100 seconds' duration resulted in definite acceleration of the heart. This increase in heart rate could have been produced only by vagal accelerator impulses.

Because of the difficulties involved in such complicated procedures as those described in the preceding paragraph, most of the experiments on the effects of acute anemia were performed with a single dog. In a typical experiment, the spinal cord was transected at the second cervical level and the highest four or five thoracic sympathetic ganglia on each side were removed. The left subclavian artery was ligated at the arch of the aorta. Heart rate and blood pressure were recorded from a cannula in the left common carotid artery. In some experiments, the lower thoracic aorta and the inferior vena cava were ligated to keep up the blood pressure in the spinal animal. Artificial respiration was carried out by means of a pump. Acute cephalic anemia was produced by clamping the right common carotid and right subclavian arteries, thus occluding all the arteries emerging from the arch of the aorta. In each experiment, in order to demonstrate the excitability of the medullary centers, acute anemia of 40 to 80 seconds' duration was produced before administration of atropine and gave rise to marked slowing of the heart through excitation of the vagus center. Then atropine was administered intravenously in doses of 0.1 mgm. per kilogram of body weight, and the brain anemia was reproduced.

Immediately after clamping the vessels to the head, no change in heart rate could be noted. After 60 seconds of anemia of the brain, definite acceleration became evident. As the anemia was continued, the heart rate increased. The usual duration of the arterial occlusion was $1\frac{1}{2}$ to 3 minutes, and at the end of this time, the average increase in heart rate was 32 beats per minute. During the period of brain anemia, there was a gradual slight rise of blood pressure. Often the head would gasp, but no respiratory movements could be observed in the trunk, demonstrating the completeness of the spinal cord transection. The lid closure reflex, which is very active under chloralose anesthesia, disappeared soon after the circulation to the brain was stopped. When the arteries were released, the blood pressure fell abruptly, usually to slightly below the original level. Cardiac acceleration, however, persisted for some time, the rate gradually decreasing and returning to the original only after 3 to 4 minutes.

Jourdan and Nowak (1936) emphasized the long persistence of cardiac acceleration after acute anemia of the brain. They reported that the heart rate remained more rapid for one to two minutes even after section of the cervical vagi. In six experiments, I produced an increase in heart rate by means of acute cephalic anemia and cut both vago-sympathetic nerves at the height of the acceleration. In every case, the heart rate was not restored to its original level after cutting the vagi until 3 to 5 minutes had elapsed. In one experiment, the vagi were sectioned coincident with restoring blood flow in the brain, yet the acceleration persisted for 4 minutes. When the anemia was continued for a minute or two after the vagus nerves were cut, the rate began to decrease from the moment of the

nerve section and continued to decrease gradually. The results of a typical experiment are shown in figure 1. These results indicate that the effect produced by vagal cardio-accelerator impulses persists for a relatively long time even after the discharge of impulses has ceased, a phenomenon characteristic of nerve endings at which sympathin is the humoral mediator.

When cephalic anemia was produced in a single animal after the vagosympathetic nerves had been sectioned, the results were very disconcerting. While in six experiments no change in heart rate was observed during acute cephalic anemia, in ten other experiments the heart rate increased during anemia even though both vagi had been cut previously. This acceleration was not inconsiderable, averaging 28 beats per minute. At first, the

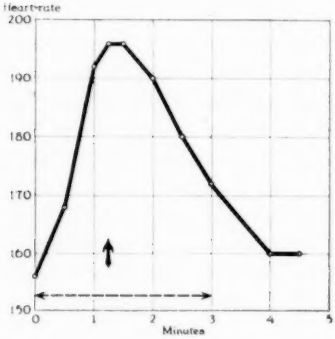

Fig. 1

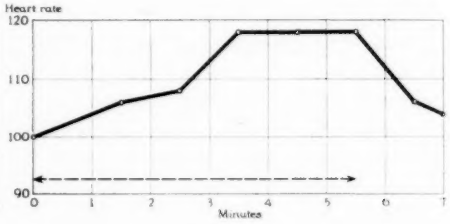

Fig. 2

Fig. 1. Effect of section of the vagus nerves during acute cephalic anemia. The horizontal line indicates the duration of the anemia and the arrow indicates the point at which the vagi were sectioned. Note the persistence of the cardiac acceleration for several minutes after section of the vagus nerves.

Fig. 2. Effect of acute cephalic anemia after a previous vagotomy. The horizontal line indicates the duration of the anemia. Note the slow increase in heart rate and the rapid recovery.

results were attributed to some fault of technique. However, careful repetition of the experiments indicated that there were two separate and distinct accelerator mechanisms, both of which became operative during anemia of the brain, but only one of which was dependent on the integrity of the vagus nerves. The other mechanism was apparently humoral.

There is little reason to doubt that with the vagus nerves intact, anemia of the brain increases the heart rate through excitation of the vagal accelerator neurons in the medulla. The strongest evidence in favor of this view is the fact that acute anemia of the isolated cross-circulated head, connected to its trunk only by the vagus nerves, produces significant cardiac acceleration. Furthermore, when the vagus nerves are sectioned

during cephalic anemia, the accelerated heart begins to slow coincident with section of the nerves, even though the anemia is continued for a time (fig. 1). Moreover, the time relations of the two types of acceleration are quite different. With the vagi intact, the heart rate increases sharply and the acceleration persists for a long time after release of the head arteries. On the other hand, after vagotomy, anemia of the brain must be greatly prolonged to produce acceleration and the increased heart rate persists for only a short time after restoration of cephalic blood flow (fig. 2). In the six experiments in which acceleration was produced by anemia with the vagi intact but not after vagotomy, the failure to increase the heart

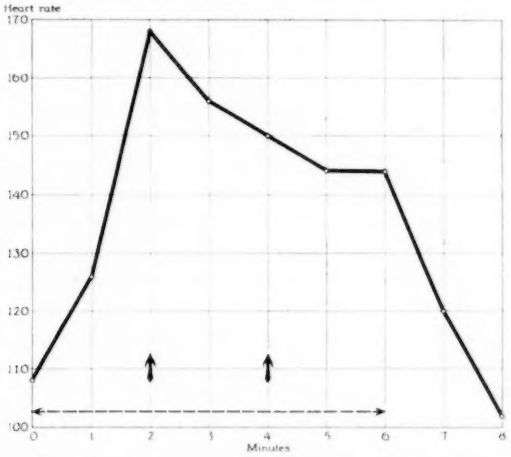

Fig. 3. Effect of section of the vagus nerves during acute cephalic anemia of relatively long duration. The horizontal line indicates the duration of the anemia. The first arrow from the left indicates section of the right vagus nerve. The second arrow indicates section of the left vagus nerve. Note the combined effect of vagal and humoral mechanism on the cardio-accelerator response.

rate after vagotomy can be accounted for by the relatively short duration of the cephalic anemia.

The interrelations of the two types of accelerator mechanisms can be studied by section of the vagus nerves during anemia of the brain. When the duration of anemia was too short to produce a significant increase in heart rate by the humoral mechanism, the heart rate decreased steadily after section of the vagi and release of the cephalic vessels produced little change in the curve (fig. 1). When, on the other hand, the anemia was maintained for a longer time (fig. 3), the heart rate decreased more slowly after section of the right vagus nerve, and even reached a plateau toward the end of the period of anemia declining quite rapidly after restoration of

blood flow. In this case, the slower decline in heart rate after section of the vagus and the rapid fall after cessation of the anemia were dependent on activation of the humoral mechanism by the long period of anemia. The slow decline in the heart rate after section of the right vagus nerve was not due to accelerator fibers in the intact left vagus, since after cutting the left vagus the heart rate declined even more slowly.

Elimination of other possible mechanisms which might account for the acceleration produced by cephalic anemia after vagotomy (which will be discussed in detail elsewhere) left only a humoral mechanism as a likely possibility. It would appear that some substance which tends to keep the heart rate slow may normally reach the heart from the brain through the blood stream. When the blood flow through the brain is stopped, the heart accelerates after a time, due to the disappearance of this humoral cardio-inhibitory factor from the blood. Very soon after blood flow through the brain is resumed, a plentiful supply of the substance is brought to the heart through the veins and the heart quickly returns to its original rate. One can only speculate at present as to the nature of this hypothetical cardio-inhibitory humor, but it seems probable that such factors as temperature, CO_2, and acid content of the blood, acetylcholine and choline do not play a significant rôle. This encephalic cardio-inhibitory substance may prove to be of importance in the determination of the "automatic" rhythm of the denervated heart.

Discussion. It is not entirely clear why the response to stimulation of the vagal accelerator fibers is so much greater after an upper thoracic sympathectomy. While it is possible that the increased effectiveness of stimulation is due to the difference in the basic heart rates before stimulation, this does not seem to provide an adequate explanation, since the average heart rate in the dogs with sympathetic innervation intact was 170 per minute while the average heart rate in the partially sympathectomized animals was 145 per minute. It is difficult to surmise what other factors may have been involved. Nevertheless, this difference in responsiveness of the vagal cardio-accelerator fibers after partial sympathectomy may help to reconcile the contradictory results obtained by certain investigators. Thus Jourdan and Nowak (1936) had no difficulty in demonstrating accelerator fibers on stimulation of the vagus nerve because they had previously performed a bilateral stellectomy. On the other hand, Hering (1924) and Kabat (1937, in a preliminary note on some of the experiments reported here) were reluctant to admit the existence of vagal cardio-accelerator fibers because the results obtained were largely negative and whatever acceleration was observed was slight. Moreover, the increase in heart rate was much greater the closer the point of stimulation to the inferior cervical sympathetic ganglion. The relative ineffectiveness of stimulation of the vago-sympathetic nerve in these experiments may be accounted for by the fact that the sympathetic innervation was left intact.

The similarity in latent period and persistence after stimulation of the responses to excitation of the vagal and of the ortho-sympathetic cardio-accelerators indicates that the same mechanism may underlie them both and that perhaps sympathin is released at the post-ganglionic endings in the heart on stimulation of the vagus fibers. Further support is given to this view by the persistence of the acceleration induced by acute anemia of the vagus center for as long as 4 or 5 minutes after section of the vagi (fig. 1). Besides, all post-ganglionic autonomic endings so far studied transmit their excitation as a result of production of either acetylcholine or sympathin, and unless we postulate some entirely new unknown mediator for the vagal accelerators, we must admit that sympathin is probably involved in the mechanism of transmission of the vagal accelerator impulses to the heart. If this mechanism is substantiated by further investigation, then one is presented with yet another example of the presence of adrenergic and cholinergic autonomic nerve fibers in the same anatomic nerve. It is evident that the old anatomic subdivision of the autonomic nervous system into sympathetic and parasympathetic systems is rapidly losing its usefulness and must give way to a more fundamental functional classification (Dale, 1934).

The threshold for stimulation of the accelerator fibers of the vagus nerve is somewhat higher than the threshold for excitation of the inhibitor innervation of the heart. This is in agreement with the observation of Hering (1924). Reed and Layman (1930) as well as some older investigators have been able to obtain cardiac acceleration in some animals by stimulation of the vagus with very weak currents without use of atropine. These investigators were probably not stimulating the vagal accelerators and the increase in heart rate must be explained in some other manner. It is also likely that the "reversal" of vagus action on the heart produced by various drugs such as nicotine, characterized by acceleration during stimulation of the vagus nerve and inhibition afterward which was demonstrated by Dale, Laidlaw, and Symons (1910) may not be due to stimulation of the vagal accelerator fibers.

Perhaps the most vexing problem in connection with the accelerator mechanism of the vagus nerve is the question of the physiological conditions of its activity. All critical attempts to excite this mechanism reflexly by stimulation of sensory nerves have failed. Anemia of the brain undoubtedly stimulates the vagal accelerator neurons but brain anemia is an uncommon occurrence and not physiological. Recently, Brouha, Cannon, and Dill (1936) and Brouha, Dill and Nowak (1937), working with sympathectomized dogs, and Hodes (1939) with sympathectomized cats, found that emotional excitement or intense exercise could increase the heart rate well above its intrinsic rhythm, often to over 200 beats per minute. It was also observed that mere administration of atropine could produce a

rapid heart rate, indicating that the accelerator mechanism involved might be in "tone." These investigators suggested, after elimination of other possibilities, that the vagal accelerator mechanism was the most important factor in this phenomenon. The critical experiment of studying emotion and exercise in a sympathectomized animal after section of the vagus nerves was performed in only one case by Hodes (1939), who found that after section of the left vagus in the neck and the right vagus below the recurrent laryngeal, exercise no longer increased the heart rate.

However, there is still some reason to doubt that emotional excitement and intense exercise activate the vagal accelerator mechanism. Thus Samaan (1935a) observed that in dogs deprived of sympathetic cardio-accelerator and splanchnic innervations, the maximal tachycardia from muscular work was 105 to 121 beats per minute. Samaan (1935b) also reported that after injection of atropine into totally sympathectomized dogs, the heart rate did not increase to more than 130 beats per minute, which is the intrinsic rhythm of the denervated heart. Moreover, Bouckaert and Heymans (1937) from experiments on sympathectomized dogs, concluded that the vagal cardio-accelerator mechanism is not activated by emotional excitement.

In an attempt to reconcile the observations of the Harvard group with those of the investigators at Ghent, Brouha, Dill, and Nowak (1937) found, strangely enough, that both groups of workers were essentially correct. If the experiment was performed soon after sympathectomy, no cardiac acceleration was observed as a result of atropine, exercise, or emotion, in agreement with Samaan (1935a, b) and Bouckaert and Heymans (1937). On the other hand, if the experiment was delayed more than three weeks after sympathectomy as was the practice of Brouha, Cannon, and Dill (1936), then the heart rate increased well above its automatic rhythm. Why should the cardio-accelerator response appear only some weeks after sympathectomy? One possibility is that the vagal accelerator mechanism is not involved in the response at all but that regeneration of sympathetic fibers accounts for it. The long delay in appearance of the cardio-accelerator response and the progressive increase in its intensity with time are suggestive of such an explanation. Since the cervical sympathetic ganglia were not extirpated in these experiments, it is possible that regenerating pre-ganglionic fibers could reach the inferior cervical sympathetic ganglion and thus accelerate the heart. However, Brouha, Dill, and Nowak (1937) and Hodes (1939) have presented functional evidence for the completeness of the sympathectomy in their animals. The other possible explanation of the phenomenon is that the vagal accelerator response normally lies dormant and only achieves its full functioning power some weeks after sympathectomy. Assuming that this is true, one is still forced to conclude that in the normal animal the vagal accelerators prob-

ably have no significance in the physiological control of the cardiac rhythm in emotion and exercise.

I should like to express my gratitude for the advice and assistance of Dr. Maurice B. Visscher in the conduct of these experiments.

CONCLUSIONS

1. Electrical stimulation of the cervical vago-sympathetic trunk and of the vagus rootlets intracranially demonstrates the existence of efferent cardio-accelerator fibers.

2. The vagal cardio-accelerator fibers are present predominantly if not entirely in the right vagus nerve.

3. The vagal cardio-accelerator mechanism cannot be excited reflexly by stimulation of the carotid sinus or of the sensory fibers of the vagus nerve or the superior laryngeal nerve.

4. Acute anemia of the brain excites the vagal cardio-accelerator mechanism.

5. Acute cephalic anemia increases the rate of the completely denervated heart by a humoral mechanism.

6. The characteristics of the accelerator response to stimulation of the vagal accelerators indicates that sympathin is probably the chemical mediator at the nerve endings in the heart.

7. The rôle of the accelerator fibers of the vagus nerve in the normal regulation of the heart rate is discussed.

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RELATIONSHIPS OF TISSUE (SUBCUTANEOUS AND INTRAMUSCULAR) AND VENOUS PRESSURES TO SYNCOPE INDUCED IN MAN BY GRAVITY[1]

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One of the commonest types of syncope is that which sometimes occurs after prolonged quiet standing. Some individuals can maintain the upright position for relatively long periods of time while others show a predisposition to syncope soon after assuming the position. In some cases, a subject may tolerate relatively long periods of quiet standing on some occasions but not on others. This type of syncope has been characterized by Lewis (1) as "vasovagal" in origin, due primarily to vasomotor depression and accompanied by a bradycardia of vagal origin. Previous studies in our laboratory (2, 3), however, have indicated that the vasomotor responses of subjects developing syncope in the upright position are not significantly impaired prior to complete collapse. They have suggested that the syncope is more probably due to a diminished venous return consequent to excessive pooling in the capillaries and veins of the dependent parts, observations which support those of Henderson (4). He has long maintained that syncope is due primarily to a diminished venous return, the result of failure of motor centers and the consequent relaxation of musculature. The present study was therefore designed to investigate the part played by muscle tonus in the development of postural syncope. The results of the experiments outlined below demonstrate that intramuscular pressure is a particularly important factor in this respect.

METHODS. Seven adult normal males have served as subjects for these experiments extending over a period of 5 months. All, except one, had served as subjects in one or more of the previous studies dealing with other aspects of the general problem (2, 3, 5). Three of the individuals, J. A. T., H. S. M. and M. L., have never shown signs of embarrassment in the upright position while two (D. R. U. and L. A. T.) have consistently shown signs of syncope. One subject, J. M. S., fainted on some occasions

[1] A preliminary report of this study was presented before the American Physiological Society at Toronto on April 29, 1939.

but not on others. The seventh subject, W. B. V., was a consistent fainter in a previous study and in the early part of this investigation. The longest period he had been able to tolerate the upright position was six minutes. During the last two experiments, however, he remained in the upright position for twenty minutes without showing any discomfort. With the exception of H. S. M. and D. R. U., all of the subjects were athletic.

In all of the experiments the position of the subjects was passively changed by the use of a tilting table. When in the upright (75°) position, the subject was supported by the buttocks resting in a shallow-grooved support bolted across the table and kept from slipping by two padded wooden blocks secured in place over the iliac crests by a cross-bar. All weight-bearing on the lower extremities was eliminated by this method of suspension. The latter requirement is of utmost importance in studies on posture since, as Hellebrandt and her co-workers (6) have shown, the center of gravity of the body as a whole shifts incessantly during natural comfortable standing and even in a relaxed subject the amount of movement may be extreme. This movement results in a marked rhythmic variation of intramuscular pressure in the gastrocnemius (7) and a lowered venous pressure in the foot veins (8, 9). Our method of suspension resulted in the elimination of practically all movement and the achievement of consistent values. By minimizing the influence of muscular movements in aiding venous return, the strain of the upright position on the circulatory system was accentuated and the consequent changes accelerated. In all experiments on fainters, signs of syncope were evident within the first few minutes and collapse was imminent within 20 minutes after being tilted. This period therefore was used as the control period for the non-fainters. The fainters were usually returned to the horizontal position just before complete collapse occurred, although, in some cases, syncope intervened before the table could be tilted back to the horizontal.

The method of Burch and Sodeman (10) was used to measure venous pressure directly in the dorsum of the left foot and in the antecubital region of the right arm. Heart level was assumed to be 10 cm. from the height of the table for the horizontal position (11) and at the fourth interspace for the upright position. Subcutaneous pressure was determined in the dorsum of the right foot and, in a few experiments, in the right leg superficial to the gastrocnemius muscle by the method of Burch and Sodeman (12). Intramuscular pressure was measured in the right gastrocnemius and right biceps by a slight modification of the method described by Henderson, Oughterson, Greenberg and Searle (13). Blood pressure and pulse rate readings were obtained from the left arm in some experiments. In a typical experiment the subject reclined on the tilting table and the various needles were inserted and anchored securely in place with adhesive strips. Simultaneous readings of two or three of the func-

tions were made by separate observers at intervals of 15 to 60 seconds. When constant levels had been established in the horizontal position, the subject was tilted and observations continued throughout the upright period and, whenever possible, after return to the horizontal position until pre-tilting levels were reached or approached. In most experiments the tilting was accomplished with no disturbance in the position of the needles. The temperature of the room in which the experiments were conducted was maintained constant at 75°F and the relative humidity at 50 per cent.

RESULTS. The average values for the horizontal position as found in the 3 types of subjects (fainters, non-fainters and intermediates) are given in table 1. The levels, in general, are similar to those of other investigators

TABLE 1

Average resting values in horizontal position

SUBJECT	VENOUS PRESSURE, CM. H_2O		SUBCUTANEOUS TISSUE PRESSURE, CM. H_2O		INTRAMUSCULAR TISSUE PRESSURE, CM. H_2O	
	Antecubital	Foot	Foot	Gastrocnemius	Gastrocnemius	Biceps
H. S. M.	12.1	24.2	4.1	5.6	10.0	4.8
J. H. T.	10.0	25.2	2.6		12.1	8.3
M. L.		27.5	1.7		11.2	
Average non-fainters	11.1	25.3	2.8	5.6	10.8	6.6
J. M. S.	11.2	20.8	2.4		5.8	8.8
W. B. V.	12.2	20.8	2.3		8.8	5.8
Average intermediates	11.9	20.8	2.3		7.6	6.8
D. R. U.	13.4	21.5	2.8	8.8	7.2	9.0
L. A. T.	9.4	21.3	2.1		3.5	4.4
Average fainters	10.7	21.4	2.5	8.8	5.4	5.9

using comparable methods. In all experiments in the horizontal position the pressure in the foot veins was higher than in the arm veins (14), the difference being particularly accentuated in the non-fainting individuals. The subcutaneous pressure of the tissue overlying the gastrocnemius as measured in 3 experiments on 2 subjects was in every case higher than the subcutaneous pressure of the dorsum of the foot. Significant differences were found in the levels of venous pressure of the foot and in the intramuscular pressure of the gastrocnemius of the three groups. While there were fluctuations in the levels of these functions there was a distinct tendency for the pressures in the non-fainting individuals to be higher than the fainting group, the "intermediate" showing values which on some

days corresponded to those of the non-fainting individuals, on other days, to those of the fainting group.

Figure 1 illustrates the changes in venous and subcutaneous pressures of the foot and in the intramuscular pressure of the gastrocnemius in a typical experiment in which a non-fainting subject was tilted from the horizontal to the upright (75°) position. Figure 2 illustrates a similar experiment on another subject in which the subcutaneous pressure was measured in the tissue of the gastrocnemius muscle at a point over that where the intramuscular pressure was determined. Figure 5 shows the changes in an experiment in which signs of syncope were marked during the upright period.

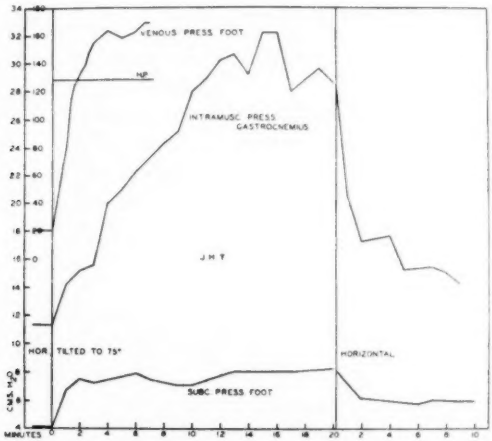

Fig. 1. Typical experiment on non-fainting subject (J. H. T.). Hydrostatic pressure (H. P.) measured to fourth interspace.

In all cases tilting was followed by an immediate sharp rise of 80 to 105 cm. H_2O in venous pressure, the value usually reaching or exceeding the hydrostatic level not later than the end of the second or third minute. Coincident with this increase in venous pressure there also occurred sharp increases in subcutaneous and intramuscular pressure. While the levels reached varied because of differences in control values, the actual increases were quantitatively similar; in the non-fainting experiments they were practically identical. One minute after tilting the foot subcutaneous pressure had increased by an average of 5.3 cm. H_2O (10 experiments on 7 subjects) while the gastrocnemius pressure rose an average of 4.2 cm. H_2O. Similar relationships obtained in several experiments in which subcutaneous and intramuscular pressures were determined simultaneously

in the gastrocnemius. The levels of venous and subcutaneous pressures reached immediately after tilting were usually maintained with minor fluctuations for the duration of the tilt period, there being no difference in the changes found in the fainting and non-fainting experiments. The course of the intramuscular pressure changes, however, differed in the two types of experiments. In the instances where signs of syncope appeared during the experiment, the intramuscular pressure curve usually followed those of subcutaneous and venous pressures. In the experiments where no embarrassment occurred, on the other hand, the intramuscular pressure showed a secondary rise (5.5 to 18 cm. H_2O) during the subsequent 10 to 15 minutes which in every case exceeded the initial increase which

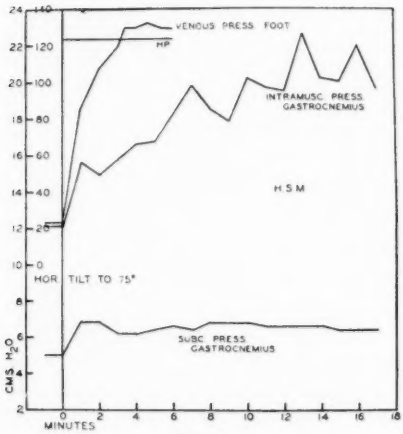

Fig. 2

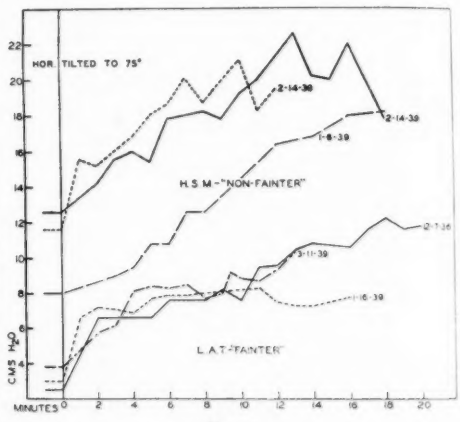

Fig. 3

Fig. 2. Typical experiment on non-fainting subject (H. S. M.) hydrostatic pressure (H. P.) measured to fourth interspace.

Fig. 3. Comparison of intramuscular (gastrocnemius) pressure changes in fainting and non-fainting subjects.

occurred immediately after tilting. In no case did the maximum values found in the fainting experiments approach the lowest values found during the tilting period in the experiments where signs of syncope were not evident (fig. 3).

Our changes in venous pressure are in agreement with those of Doupe, Krynauw and Snodgrass (15) who also used the fourth interspace as the zero pressure level in the right auricle in the upright position. In no experiment did the venous pressure ever fall below hydrostatic pressure as measured to this reference point. In the experiments in which syncope did not develop, the venous pressure as measured in the foot veins exceeded the hydrostatic level by 10 to 17 cm. H_2O. In one experiment on

subject J. A. T. the venous pressure was 40.9 cm. H_2O above hydrostatic level at the end of the seventh minute in the tilted position (fig. 1). In the fainting experiments, however, the pressure was always within 1 to 2 cm. H_2O of the hydrostatic pressure just before collapse was imminent. These findings differ from those of McIntire and Turner (14) and Weiss, Wilkins and Haynes (16) who reported values below hydrostatic level in postural syncope. The discrepancy is due, in part, to the difference in reference points. The former investigators made no mention of the reference point used; the latter used the second interspace. Both groups determined venous pressure by the indirect method. Their values should, therefore, be increased by about 10 cm. H_2O, the value of subcutaneous pressure in the dorsum of the foot in the standing position (10). In the absence of muscular movement and in the presence of venous congestion it would seem extremely improbable that venous pressure in the foot veins could fall below hydrostatic level.

That the level of pressure of the leg muscles is an important factor in the development of syncope was indicated by the experiments on the "intermediate" subjects. Two experiments on J. M. S. are illustrated in figure 4. On both days, the initial levels in the horizontal position were similar. In the experiment performed on January 14, however, the intramuscular pressure rose steadily so that at the end of 11 minutes it was at 12.2 cm. H_2O. At this point the subject complained of slight dizziness and became somewhat restless. The slight, more or less involuntary, movements which occurred at this time resulted in a rise in pressure to a peak of 15.0 cm. H_2O, followed by a subsequent fall. The dizziness disappeared during this period and the subject remained comfortable until the 28th minute when he again complained of being dizzy. Again the movements, now more pronounced, resulted in a rise in pressure to 15.8 cm. H_2O, and the subject reported "feeling fine" at the end of 34 minutes. In contrast, on January 28, fourteen days later, the pressure rise which occurred immediately after tilting was not maintained, so that at 10 minutes the subject complained of dizziness, which increased and at 16 minutes was accompanied by nausea and belching. As in the previous experiment, the incident restlessness resulted in an increase in pressure but the level reached was only 11.4 cm. H_2O. At 20 minutes the subject was at the point of collapse and was returned to the horizontal position.

The results of these experiments suggested that syncope might be prevented in the fainters by the simple expedient of increasing their intramuscular pressure. The usual method of support was, therefore, modified by eliminating the supports over the iliac crests and allowing the subjects to stand on their toes on a support fixed to the lower edge of the tilting table. Such an experiment is illustrated in figure 6. The

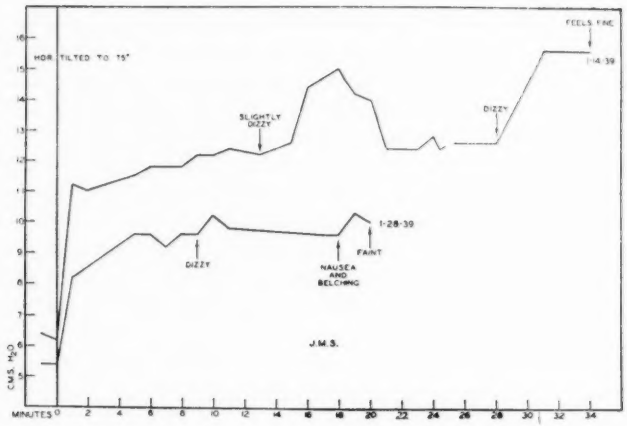

Fig. 4. Variations in intramuscular (gastrocnemius) pressure and in development of syncope in same subject on different days.

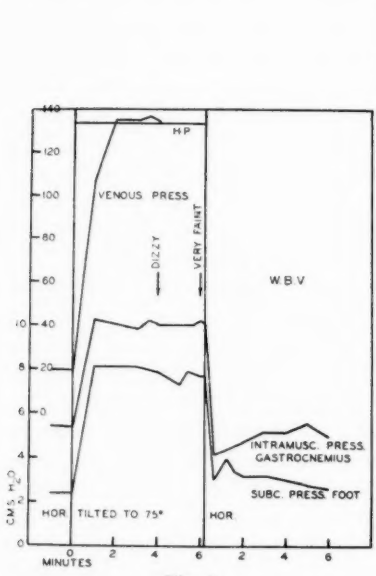

Fig. 5

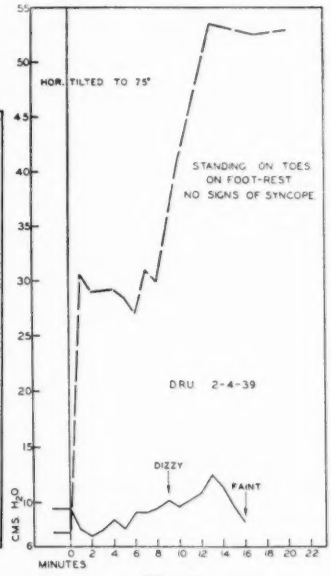

Fig. 6

Fig. 5. Typical experiment on fainting subject (W. B. V.). Hydrostatic pressure (H. P.) measured to fourth interspace. Subject at point of collapse at end of 6 minutes in upright position.

Fig. 6. Relationship of intramuscular (gastrocnemius) pressure to syncope. Details given in text.

lower curve shows the first experiment in which the subject was supported in the usual manner and was at the point of collapse at the end of 16 minutes. He was then returned to the horizontal position, the supports removed and a foot-board adjusted to the tilting table. After a rest period of 13 minutes in the horizontal position he was again tilted, standing on his toes, but with as little movement as was possible. The upper curve shows the pressure changes. The sharp rise at 10 minutes was occasioned by his getting up further on his toes. None of the usual signs of syncope appeared and the experiment was concluded at the end of 20 minutes. In a similar experiment on subject W. B. V. the intramuscular pressure in the gastrocnemius rose to beyond 74.8 cm. H_2O, this being the limit of the manometer in use. As long as he remained on his toes and the intramuscular pressure was high, no signs of syncope were manifest. When he was allowed to stand "flat" on his heels the pressure fell rapidly and he complained of being dizzy.

The experiments on W. B. V. are of further interest in this connection. Attempts to use this subject in a previous study were unsuccessful because of his inability to tolerate the upright position for more than a minute or two. During the months of December and January of the present study, he was suspended in the upright position on 4 separate occasions. His intramuscular pressures before tilting varied from 5.6 to 11.5 cm. H_2O on the different days and the maximum value reached in any one experiment was 17.6 cm. H_2O. The longest period he was able to remain upright was 6 minutes (fig. 5). He was not used in any experiments during February, but was the subject of an experiment on March 1, in which the venous pressure in the arm and intramuscular pressure of the biceps were studied. He was then able to tolerate the upright position for 20 minutes with none of the usual signs of syncope being present. A subsequent experiment on March 4, showed the pressure in the gastrocnemius to be 18.7 cm. H_2O in the horizontal position. Again he was able to maintain the upright position for 20 minutes, the intramuscular pressure rising to a maximum level of 23.6 cm. H_2O by the fifteenth minute. Questioning revealed the fact that the subject was experiencing considerable emotional excitement during this period.

Discussion. Henderson et al. (13) suggested that intramuscular pressure varied with muscle tonus, a concept which has been challenged by Wells, Youmans and Miller (17). These authors believe that intramuscular pressure is determined primarily by the tightness of the overlying fascia, the amount of extravascular fluid and the degree of filling of blood vessels. Hellebrandt, Crigler and Kelso (7), on the other hand, have recently affirmed the validity of the intramuscular pressure method as a measure of tonus and concluded "there is little doubt that as the contraction of motor units become more synchronous the pressures accrue suf-

ficiently to reach values much in excess of the normal turgor present in the tissues. It is probable that in short time experiments concerned with relaxed and comfortable standing, the number of motor units contracting and the synchronicity of their response are factors of greater importance in the production of intramuscular pressure changes than stasis or edema."

Of particular interest in this connection are the relationships of venous, subcutaneous and intramuscular pressure curves in our fainting and non-fainting experiments. The immediate sharp increases in subcutaneous and intramuscular pressure observed in all cases when the position was changed are seemingly due to venous congestion. This is borne out by the quantitative similarity of the changes in the two pressures and by the fact that there is an approximate correlation between the amount of venous pressure increase and the increases in subcutaneous and intramuscular pressure. The secondary rise in intramuscular pressure observed in non-fainting experiments (figs. 1 and 2) is a manifestation of the superimposed tonic influence of the muscle fibers. When, as in the fainting experiments, the tonus does not increase, the intramuscular pressure curve parallels that of subcutaneous pressure and does not show the secondary rise (fig. 5). These results afford a probable explanation of the difference in the levels of intramuscular and subcutaneous pressures in the relaxed, horizontal position. The higher values usually found for intramuscular pressure in the non-fainting experiments can be explained as being due to the fact that in addition to the pressure exerted by extravascular fluid and the contents of the blood vessels, there is the component due to the tonus of the muscle fibers. When tonus is low (table 1) the values for intramuscular pressure tend to approach those for subcutaneous pressure. When tonus is absent, intramuscular pressure is primarily determined, as Wells, Youmans, and Miller (17) suggest, by the tightness of the overlying fascia, the amount of extravascular fluid present in the muscle, and the degree of filling of its blood vessels.

Our experiments fully substantiate the contention of Henderson (4) that muscles give support to the veins and that in the absence of muscle tonus, the venous return may be seriously impaired and syncope result. Individuals who show a tendency to faint when relaxed in the upright position are immediately relieved of all discomfort if allowed to increase their muscular tone by slight movement of the lower extremities. This has long been empirically recognized in instructions commonly given to students in military schools to "walk" in their shoes and to stand on the balls of their feet during parade. The intramuscular pressure of the gastrocnemius can be raised to surprisingly high levels when the weight of the body is held on the toes. Henderson (13) reported that intramuscular pressure in the gastrocnemius rose from 14.3 to 31.2 cm. H_2O

under these conditions while Hellebrandt, Crigler and Kelso (7) observed it to rise from 28.5 to 33.8 and from 23.0 to 34.3 in two similar experiments, increasing to 40.1 and 40.7 respectively when the knees were fully extended. The values attained in our experiments are considerably higher. In two similar experiments on different subjects the pressure rose from 26.3 to 52.9 and from 13.8 to over 74.8, respectively. The maximum values could not be accurately obtained in the second experiment since the limit of the manometer had been reached. Even these high pressures are still below the venous pressure in the leg and would be insufficient to completely collapse the veins, particularly since maximum values for venous pressure are reached before those for intramuscular pressure. It is probable, as Wells, Youmans and Miller concluded, "that veins can be only partially collapsed by a single contraction and that more than one contraction will be required to completely empty a section of vein below a competent valve."

Our studies would lead us to believe that in the syncope induced by prolonged standing at least, failure of muscular contraction is the primary factor leading to a secondary failure of the circulation. During the period preceding collapse, the increased diastolic pressure, decreased tissue temperatures, pallor, tachycardia and sweating which are invariably present, attest the fact that the vasomotor and sympathetic centers are being intensely stimulated rather than depressed. The available evidence suggests that one of mechanisms for adjustment to the upright position is a vasoconstriction and slowing of the volume flow in the dependent parts, thus preventing a flooding of the capillary reservoirs. Such a compensatory mechanism is only partially effective, however, for once the blood succeeds in getting through to the veins, it has difficulty in returning to the heart as manifested by a marked increase in circulation time and by a decrease in cardiac output. In this way a vicious cycle is created. The volume flow is slowed not only as a result of the difficulty in returning blood to the heart against gravity but also by the compensating mechanism itself. If muscle tonus is low, there will therefore be a greater tendency for stagnation to occur and for the venous flow to be slowed to such an extent as to fail to meet the demands of gas transport for the individual. The weakness which leads to syncope is thus not on the arteriolar, but on the venous side of the system. Smith and his associates (18) have recently emphasized this point of view in explanation of the hypotension of spinal anesthesia.

Our data show that muscle tonus varies in different individuals and from time to time in the same individual. Subjects who show a low level of muscle tonus in the horizontal position invariably have greater difficulty in maintaining the upright position without signs of circulatory embarrassment than those who show a higher tonus. Likewise, no discomfort is

experienced in the upright position when the tonus is high, but signs of distress appear when the tonus drops or remains low. These relationships were so definite in our experments as to make it possible to predict the behavior of the subject before or within the first few minutes after he was tilted. Any explanation for the differences in muscle tonus can only be conjectural at this time. However, the mere fact that the same normal individual may show relatively great variations on separate occasions makes it difficult to explain the differences on a strictly anatomical basis. Such variations must be primarily related to functional factors. The observations on W. B. V. suggest that the psychic state of the individual is of utmost importance.

SUMMARY

1. Venous (foot) and intramuscular (gastrocnemius) pressures in the resting, horizontal position are lower in individuals who develop postural syncope than in those who do not.

2. Tilting of individuals from the horizontal to the upright (75°) position is followed by immediate and simultaneous rises in venous, subcutaneous and intramuscular pressures. Subjects who do not develop syncope show a secondary, usually more marked, increase in intramuscular pressure during the upright period, a change which is absent in those who show circulatory embarrassment.

3. Intramuscular pressure is affected by the tonus of the muscle fibers as well as the amount of intravascular and extravascular fluid present. When tonus is low, the value for intramuscular pressure tends to approach that of subcutaneous pressure.

4. Syncope does not occur in the upright position if any significant amount of tonus is evident. Likewise, increasing the tonus by muscular contraction eliminates all signs of syncope.

5. The development of syncope in the upright position is due primarily to a diminished venous return which leads secondarily to vasomotor failure.

We are indebted to Messrs. W. B. Virgin and M. Levy for their technical assistance and to the medical students and staff members who served as subjects for these experiments.

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THE IDENTITY OF THE GLYCOTROPIC (ANTI-INSULIN) SUBSTANCE OF THE ANTERIOR PITUITARY GLAND

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It has been known for some time that injection of anterior pituitary extracts into normal and hypophysectomized animals will render the animal resistant to the hypoglycemic action of insulin, administered several hours after injection of the pituitary extract. The literature concerning this subject has been reviewed recently (1, 2). Young (3, 4) has investigated the anti-insulin action of various anterior pituitary preparations and proposed the term "glycotropic" for this effect. He found that the pituitary substance which inhibits the hypoglycemic action of insulin (glycotropic factor) was different from prolactin, the diabetogenic principle, the thyrotropic factor, and the gonadotropic hormones. The exact identity of the glycotropic factor of the anterior pituitary, however, still remains an unsettled question.

It has been demonstrated by Britton and his associates (5), and by Long and his associates (6), that the secretion of the adrenal cortex will produce an increase in blood glucose and in liver glycogen of both fed and fasted normal animals. Bennet (7) observed hyperglycemia and increased liver glycogen values in hypophysectomized rats upon prolonged administration of the adrenotropic hormone. It occurred to us, therefore, that the glycotropic effect of the anterior pituitary may be due to the adrenotropic hormone. In order to investigate this possibility we tested various purified anterior pituitary preparations and also the secretory product of the adrenal cortex and that of the thyroid for glycotropic activity. From the results recorded below it appears that the adrenotropic factor is responsible for the glycotropic effect of the anterior pituitary.

EXPERIMENTAL. The glycotropic effect of the various hormone preparations was studied in mice, in general following the procedure outlined by Campbell (8). Male mice, about 20 to a group and ranging in weight from 18 to 22 grams, were injected subcutaneously at the onset of a 6 hour fast with 0.5 cc. of a neutral or slightly alkaline solution of the preparation being tested. During this period the animals were kept at room temperature with access to water. At the end of the fasting period

each animal received a 0.25 cc. subcutaneous injection of 0.03 or 0.04 unit of insulin (1.5 or 2 units per kilo) and they were then placed in a specially designed thermostat for insulin assay work, the temperature being maintained at 34°C.[1] In all instances a control group of animals receiving insulin only was included in the experiment. The number of convulsions occurring during the $1\frac{1}{2}$ hour period following insulin injection was determined for both control and test groups. The results of the various experiments are shown in tables 1 and 2. In the control groups 80 to 90 per cent convulsions were generally observed. We purposely gave a large dose of insulin to insure a high percentage of convulsions in the controls in order to make more evident the protective action of those substances demonstrating the anti-insulin effect. The percentage of convulsions in those groups of animals receiving adrenotropic or adrenal cortical preparations was significantly low, indicating the glycotropic nature of these hormones, whereas the other principles investigated failed to bring about a decided decrease in the number of convulsions as compared with the controls.

The various substances tested for glycotropic activity were prepared and standardized as follows:

a. *Prolactin* was prepared according to the method of Lyons (9), the M.E.D. of the final product being between 0.1 and 0.2 mgm., employing the systemic 4-day test in squabs. The activity of the preparation used in our work was of about the same magnitude as that reported for crystalline prolactin (10).

b. *FSH* was prepared and standardized according to the method recently published by Jensen, Simpson, Tolksdorf and Evans (11). The M.E.D. of the preparation used in our experiments was 0.05 mgm.

c. *Forty per cent alcohol extract* of whole sheep pituitary was prepared and standardized according to the method of Jensen et al. (11). It has been found that such a preparation contains the FSH, ICSH (LH, thyrotropic), and adrenotropic factors. It has not been tested for prolactin content.

d. *Thyrotropic*—Investigations carried out in this laboratory point to the identity of the ICSH and thyrotropic A.P. factors. The thyrotropic preparation A represents a main ICSH fraction prepared according to Jensen et al. (11) and was found to contain 4 chick units of thyrotropic activity per milligram as determined by the method of Smelser (12). A chick unit is defined as the total amount of hormone which, injected subcutaneously into 4 day-old chicks, twice daily for 4 days, will show a minimal stimulation of the thyroid gland by the fifth day after the onset of injection, as determined histologically. The thyrotropic activity has

[1] We are greatly indebted to Mr. H. A. Holaday and his staff of the Biological and Development Laboratory, for placing this apparatus at our disposal.

also been checked in hypophysectomized immature female rats. Thyrotropic preparation B was prepared according to the method of Lambie and Trikojus (13) and contains 10 guinea pig units per milligram. Thyrotropic preparation C was prepared according to the procedure of Junkmann (14) and also contains 10 guinea pig units per milligram.[2]

We generally observed that when the thyrotropic preparation showed a pronounced anti-insulin effect (such as the Junkmann preparation C) it was still contaminated with a certain amount of adrenotropic hormone as indicated by tests made in hypophysectomized immature female rats. In order to remove the adrenotropic factor the following procedure was employed. The preparation was extracted with 0.2 saturated ammonium sulfate at pH 6.5 and the thyrotropic factor precipitated from the supernatant by making it half saturated with ammonium sulfate. After removal of the salt by dialysis, the thyrotropic principle was precipitated in 85 per cent alcohol. The thyrotropic material thus obtained was generally found to be free from contamination with the adrenotropic factor. Preparation D was made in this manner.

e. *Adrenotropic* was prepared according to the method of Lyons (9) and standardized according to Moon (15). We also checked the activity of our preparations in immature hypophysectomized rats. Our preparation adrenotropic A contains one Moon unit per 10 mgm., and adrenotropic B one unit per 20 mgm.

f. *Cortical preparations.* The Swingle cortical extract was prepared in the laboratory of Dr. W. W. Swingle, at Princeton University and assayed 60 dog units per cc. It was found to be free of epinephrine. The Kendall extract contained 8 dog units per cubic centimeter. In testing both these extracts the total amount was administered subcutaneously in four injections at 0, 1, 2 and 3 hours from the onset of the fast, thus allowing three hours between the last injection and the insulin treatment. This procedure was adopted because we found that the extracts were ineffective when given in one injection at the commencement of the fasting period, probably due to the rapid rate of absorption and excretion.

The crystalline corticosterone acetate used in our work was kindly supplied by Dr. E. C. Kendall. This material was dissolved in corn oil, 0.2 cc. containing 0.5 mgm. of the substance, and was given in one subcutaneous injection at the onset of the six hour fast. When testing the anti-insulin effect of this material the control group always received a like amount of corn oil alone which, however, failed to influence the insulin sensitivity of the animals.

The desoxycorticosterone acetate used in our studies was kindly sup-

[2] Preliminary biological standardizations indicate that one chick unit corresponds approximately to 2.5 guinea pig units.

plied by Dr. E. Schwenk. It was administered in the same manner as corticosterone acetate.

g. E. R. Squibb and Sons' crystalline *thyroxine* was used in this study and like the cortical extract was administered in four doses over the first three hours of the fasting period.

Discussion. The results of the present investigation, carried out in mice, indicate that the adrenotropic factor is responsible for the glycotropic (anti-insulin) effect of anterior pituitary extracts. The exact mechanism whereby the anti-insulin action of the adrenotropic principle is exerted is not clear at present. Since the data concerning the influence of the pituitary on metabolism are still contradictory we prefer not to discuss at present the possible physiological significance of our findings. The

TABLE 1

Anti-insulin tests with 1.5 units of insulin per kilo

PREPARATION INJECTED	TOTAL AMOUNT PER ANIMAL	TOTAL NUMBER OF ANIMALS	NUMBER OF CONVULSIONS	PER CENT CONVULSIONS
Controls		137	97	71
40 per cent alcohol extract	10.0 mgm.	40	2	5
Adrenotropic A	2.0 mgm.	20	8	40
Adrenotropic A	3.0 mgm.	25	0	0
Cortical extract Swingle	20 dog units	25	7	28
Cortical extract Swingle	40 dog units	25	9	36
Cortical extract Swingle*	20 dog units	20	2	10
Cortical extract Swingle*	40 dog units	19	0	0
Lactogenic	1.0 mgm.	20	12	60
Thyroxine	0.05 mgm.	20	19	95

* Total dose administered in 4 injections at 0, 1, 2 and 3 hours from onset of 6 hour fast.

exact biological relationship of the glycotropic, glycostatic, and diabetogenic effects produced by the anterior pituitary requires further investigations on a quantitative basis.

Our findings, that the injection of prolactin, thyrotropic preparations (free of the adrenotropic factor), and FSH will not inhibit the hypoglycemic action of insulin are in accord with similar observations reported by Young (4). The observation that the administration of desoxycorticosterone acetate, at both 0.5 mgm. and 1 mgm. levels, failed to demonstrate a glycotropic effect is somewhat surprising since it has been reported by various investigators that the synthetic product is more successful in maintaining adrenalectomized animals in a healthy condition than is corticosterone itself. We are unable to give an explanation for this

difference in behavior of the two substances but think it may be due to unequal rate of absorption and excretion. We are trying, at present, to determine whether, under different experimental conditions, a glycotropic effect of desoxycorticosterone acetate can be demonstrated.

TABLE 2

Anti-insulin tests with 2 units of insulin per kilo

PREPARATION INJECTED	TOTAL AMOUNT PER ANIMAL	TOTAL NUMBER OF ANIMALS	NUMBER OF CONVULSIONS	PER CENT CONVULSIONS
Controls		325	287	88
40 per cent alcohol extract	10.0 mgm.	41	9	22
Adrenotropic A	3.0 mgm.	18	7	39
Adrenotropic B	5.0 mgm.	97	14	14
Cortical extract Kendall*	16 dog units	6	0	0
Cortical extract Swingle*	20 dog units	17	12	70
Cortical extract Swingle*	40 dog units	53	18	34
Corticosterone acetate	0.25 mgm.	19	4	21
Corticosterone acetate	0.5 mgm.	46	3	7
Desoxycorticosterone acetate	0.5 mgm.	30	26	87
Desoxycorticosterone acetate	1.0 mgm.	15	12	80
Lactogenic	5.0 mgm.	52	42	81
FSH	5.0 mgm.	31	26	84
Thyrotropic A	5.0 mgm.	47	29	61
Thyrotropic B	5.0 mgm.	14	11	78
Thyrotropic C	5.0 mgm.	18	9	50
Thyrotropic D	5.0 mgm.	30	21	78
Thyroxine*	0.5 mgm.	51	47	92

* Total dose administered in 4 injections at 0, 1, 2 and 3 hours from onset of 6 hour fast.

SUMMARY

The insulin resistance of mice after the injection of various hormone preparations has been determined. We found that adrenotropic A.P. fractions as well as adrenal-cortical extracts and crystalline corticosterone acetate produce a definite "glycotropic" effect. On the other hand, administration of purified thyrotropic preparations (free from adrenotropic hormone), prolactin, and FSH preparations, as well as crystalline thyroxine fail to render the animal resistant to insulin under the experimental conditions employed. The results suggest that the glycotropic effect of the anterior pituitary is produced by the adrenotropic factor.

We wish to express our appreciation to Dr. E. C. Kendall of the Mayo Clinic for kindly supplying us with cortical extract and crystalline corticosterone acetate. We are also indebted to Dr. E. Schwenk of the

Schering Corporation for supplying us with synthetic desoxycorticosterone acetate and the thyrotropic preparation C (Junkmann).

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AN ATTEMPT TO DETECT REFLEX CHANGES IN BRONCHIAL CALIBRE SYNCHRONOUS WITH RESPIRATION[1, 2]

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That the calibre of the bronchi increases during inspiration and decreases during expiration is well known. Although these calibre changes might readily be explained on a purely passive basis the view is widely held that they are in part at least the result of rhythmic contractions of the bronchial musculature. Macklin in his comprehensive review on the musculature of the bronchi and lungs (1929) suggests that the afferent vagal impulses which are initiated by distention of the lungs and which play a part in terminating inspiration may also stimulate the center in the vagus nucleus which causes the bronchial muscles to contract. "Hence, when the chest wall begins its relaxation, the lung finds a muscular tube beginning to contract, and accommodating itself in size and length to the diminishing size of the lung. Expiration, passive in the external muscles of respiration, may be looked upon as the active phase in the bronchial tree. . . . The vagus is looked upon as the motor nerve of the bronchial musculature and many experiments support this." Although this concept of an active contraction of the bronchial musculature with each expiration has gained wide acceptance it apparently rests almost entirely upon an anatomical and teleological basis. A survey of the literature reveals very little experimental evidence supporting it or directly bearing upon it.

The experiments here described were designed to test the hypothesis that the lungs might be the source of afferent impulses responsible for rhythmic contractions of the bronchial musculature. Our experimental method was based upon the supposition that if the two lungs were ventilated simultaneously the reflexes arising in the two lungs would mutually reinforce, a reinforcement which would be absent if inspiration in one lung was synchronous with expiration in the other. Dogs anesthetized with morphine and urethane or more frequently with chloralose were used. The cannula shown in figure 1, similar in principle to one described

[1] These experiments were supported by a grant from the Rockefeller Foundation to Robert Gesell for studies on respiration.

[2] Preliminary Report: Proceedings, American Physiological Society, This Journal **123:** 205, 1938.

by Head (1889), was inserted through the trachea, the curved end extending into the left main bronchus. The cannula was provided with a rubber cuff inflation of which completely closed the bronchus proximal to the opening of the cannula. A manometer was connected to the cuff to eliminate any uncertainty regarding the maintenance of the pressure within it. An artificial respiration pump was connected to each side of the cannula to permit ventilation of the two lungs independently. Our usual method of recording changes in bronchial calibre was based upon that described by Jackson (1917). During artificial respiration ribs 3, 4, 5, 6, 7 and 8 on both sides, including the portion of the sternum to which they were attached were resected as far laterally as the region of the costal angle. A metal box, illustrated in figure 2, was then carefully placed about each lung. This container, of sheet brass, was made in two parts, upper and lower, which after insertion of the lung were tightly

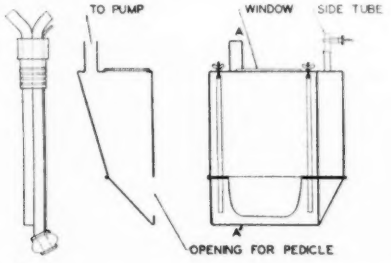

Fig. 1 Fig. 2

Fig. 1. Bronchial cannula.

Fig. 2. Container in which lung was placed. Right, a view of the medial surface of the container used for the left lung. Left, a section along line AA'.

clamped together and sealed with wax. The space around the pedicle was closed with a rubber dam fastened to the edges of the opening, and with vaselined gauze. The lung could be inspected at any time through a glass window in the top of the box. When the lungs had been placed in the containers each respiration pump was disconnected from the bronchial cannula and connected to one of the containers through tube A, a valve on the pump having been turned so that air was rhythmically withdrawn from the box. The air withdrawn from the box could be replaced in two ways—by entrance of air through the side tube or by expansion of the lung. The stroke of the pump and the resistance of the side tube could be varied. A manometer connected by a T-tube with tube A recorded the extent of the negative pressure being used to inflate the lung. Adequate inflation could ordinarily be produced by a negative pressure of 5 to 10 mm. Hg. Expiration occurred passively due to the elasticity of the lung.

The degree of inflation of the lung would of course be determined by the resistance offered by the respiratory passages as compared with that offered by the side tube. A bronchial constriction would result in a lessened inflation of the lung. In some of the earlier experiments the degree of inflation of the lung was recorded, as described by Jackson, by a large-bowled tambour connected by T-tube to the bronchial cannula, the other end of the cannula being partially closed. In most of the experiments the lung inflation was recorded by a small Hutchinson spirometer connected to the bronchial cannula.

In a few experiments the method described by Tiefensee (1928) for detecting changes in bronchial calibre was used. In this method a weighted spirometer is connected by T-tube to the bronchial cannula. A pump rhythmically forces a constant volume of air into the bronchial cannula, some of the air entering the lung and some the spirometer. A bronchial constriction shows itself by an increase in the spirometer excursion.

When the lungs had been enclosed in the containers and the two bronchial cannulae connected to their respective spirometers or tambours the two pumps were adjusted to slightly different rates, the ventilation usually being sufficient to produce apnea. If a reflex inhibition of the bronchial musculature occurred on inspiration and a reflex constriction on expiration we should expect that when the two pumps were in phase the lung inflation should be greatest, when they were in opposite phases the inflation should be least. The spirometer records should show then a series of waves, the crests appearing when inspiration occurred simultaneously in the two lungs, the troughs when inspiration in one lung coincided with expiration in the other. *In a series of over thirty experiments we have been unable to demonstrate the occurrence of any such rhythmic reflex contraction of the bronchial musculature.* It is true that the spirometer or tambour excursions did not always show a perfectly constant amplitude. Spontaneous changes in amplitude occurred which might be interpreted as due to changes in tonus of bronchial muscle. In a few experiments, one of which is illustrated in figure 3, rhythmic changes in amplitude were seen with the duration of the waves corresponding to the difference in frequency of the two pumps. However, such waves when they were observed were invariably found to be unaffected by section of both vagi or removal of both thoracic sympathetic trunks. In one experiment they continued undiminished an hour after the death of the animal. They obviously then were not reflex in origin but were presumably the result of a slight leak past the cuff of the bronchial cannula or some similar mechanical factor.

Obviously negative results mean nothing unless the method is shown to be capable of detecting changes should they occur. That our method

was sensitive is shown in figure 4, where it is seen that peripheral vagal stimulation and pilocarpine cause a marked bronchial constriction, the pilocarpine effect being promptly abolished by atropine. In the record at the left a spirometer was used for recording respiratory volume, upstroke representing inspiration. In the record at the right a tambour was used, downstroke representing inspiration. The middle record showing the effects of pilocarpine and atropine was from one of the few experiments in which Tiefensee's method was used. With this method a decreased inflation of the lung due to constriction of the bronchi is indicated by an increased excursion of the spirometer, with downstroke representing inspiration. This method seemed to be somewhat less sensitive than the one which we more commonly used. In the majority of our experiments vagal block was without effect though in about twenty per cent

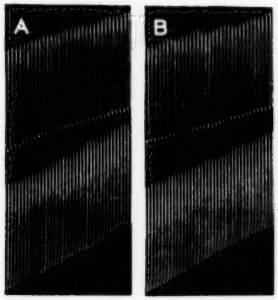

Fig. 3

Fig. 4

Fig. 3. Spirometer records of the ventilation of the two lungs before (A) and after (B) double vagotomy. The upper record is from the right lung, the lower from the left. The upper and lower records are exactly synchronous.

of the trials there was some evidence of bronchial dilatation suggesting that ordinarily the vagus exerts relatively little tonic influence on the bronchial musculature. In a large number of trials we were unable to demonstrate a change in bronchial calibre from stimulation of the central end of the vagus. These results of vagal block and central stimulation are in agreement with the early findings of Einthoven (1892) and Dixon and Brodie (1903).

Our conclusion, that the rhythmic changes in bronchial calibre synchronous with respiration are not reflex but are probably purely passive is in agreement with the view of Ellis which was based upon experiments in which he recorded changes in calibre of one of the larger bronchi. We are however unable to accept his belief that the tone of the bronchial muscle prevents collapse of the smaller bronchi on expiration, since contraction of the muscle would favor occlusion of the lumen rather than

preventing it. We can see no reason for assuming the active participation of the bronchial muscle in rhythmic respiration on teleological grounds as suggested by Macklin and others, as eliminating this type of activity by no means leaves the bronchial musculature functionless. A contraction of the bronchi as a result of the inhalation of irritating gases has been shown to occur (Dixon and Brodie, 1903) while peristaltoid movements are doubtless of the greatest importance in the removal of exudate. Finally, when one considers the characteristics of smooth muscle contraction and the extreme variations in respiratory frequency which may occur it is very difficult to conceive of the bronchial muscle actively participating in each respiration. Certainly no comparable example of such a synchronization of activity in smooth and skeletal muscle is available.

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THE CALORIGENIC ACTION OF ADRENALIN: PROPORTIONALITY WITH DOSE

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Although the calorigenic action of adrenalin has received considerable attention the particular aspect of it under consideration here, proportionality with rate of intravenous injection, seems hitherto to have escaped systematic exploration.

These data are third in a series of which changes in blood sugar and lactic acid have already been described (Griffith, Lockwood and Emery, 1939); they will show that, under the experimental conditions employed, adrenalin increases oxygen consumption maximally when injected in moderate amount, higher rates of injection having increasingly less effect; thus resembling the hyperglycemia more than the simultaneous changes in blood lactic acid concentration produced by these same injections in the same series of animals.

PROCEDURE. Cats were used exclusively and were under chloralose anesthesia; they were undisturbed except for insertion of: a tracheal cannula; a cannula in each carotid (one for blood sampling, the other for blood pressure); and a cannula in a superficial branch of the femoral vein for injection. These operations, begun as soon as anesthesia was complete, were followed by a rest period of at least 15 to 20 minutes for stabilization before observations were begun.

Animal temperature. During this period of preparation careful attention was given to stabilization of rectal temperature at an approximately normal level by means of an electrically heated animal holder and adding or removing appropriate coverings.

Determination of the respiratory exchange was effected by collection and analysis of expired air. Valves attached to the tracheal cannula directed expired air into a sensitive and carefully calibrated 2.5-liter spirometer. A by-pass inserted between the expiratory valve and the spirometer permitted coupling with a calibrated, mercury-filled, gas-sampling bulb. During any experimental period the bulk of the expired air was directed into the spirometer for measurement; at the same time an aliquot was drawn into the sampling bulb by gradual and carefully regulated fall of

its mercury, thus securing sufficient sample for duplicate analysis with Haldane apparatus.

To eliminate as far as possible error from variable operation of the spirometer, such as might result from unsuspected faults of calibration or balancing, the prevailing custom was to note the time required for it to fill between marks. This interval for any fixed volume would vary according to the size and condition of the animal; in order to keep it as nearly 5 minutes as possible the volume to be employed throughout the experiment with any one cat was selected after preliminary trial and thereafter adhered to.

Usually two determinations of the normal resting respiratory exchange, 15 to 20 minutes apart, were made before injection.

Since injection was always for exactly 5 minutes, and collection of expiratory sample was adjusted to take about this same amount of time, collection was begun co-incidentally with injection and was approximately co-terminous with it; thus determining the average respiratory exchange during most of the 5-minute injection period.

Post-injection, two determinations were made covering approximately the intervals 5 to 10 minutes, and 25 to 30 minutes after injection was completed.

Injection. Parke-Davis adrenalin chloride was diluted with neutral (pH 7.3) isotonic NaCl solution so the amount to be injected per minute was contained in 1 cc. Injection into the femoral vein was always for 5 minutes, 1 cc. per minute, by hand from a 5 cc. syringe with care to maintain uniformity of rate. The rates employed, with the number of experiments (parenthesis) were, milligrams per kilo per minute: 0.00025 (10); 0.00050 (10); 0.00100 (9); 0.00200 (14); 0.00400 (12); 0.00700 (10); 0.01000 (9); total number of injections, 74.

The experiments were begun with the intermediate rates of injection and later extended in the direction of both extremes. The results indicated little would be obtained with rates lower than the above minimum. At the other extreme, 0.01 mgm. per kilo per minute was fatal for two cats out of twelve; although the remaining ten (of which metabolism data were secured on only nine) survived with no evidence of deleterious effect, nothing of physiological significance could be anticipated from employment of rates of injection beyond this apparent maximum of tolerance.

Control injections of isotonic NaCl (10 experiments) were similar in amount, rate and duration.

RESULTS. *Normal Resting Oxygen Consumption. Intra-individual stability:* with 57 of the 84 animals used, two determinations of the "normal," resting respiratory metabolism, 15 to 20 minutes apart, were made preceding injection. The average of the first is 6.35, and of the second, 6.24 cc.

oxygen per kilo per minute. That is, on the average, injection is superimposed upon a practically constant metabolic rate; the oxygen utilization, in this respect, resembling more closely the constant blood sugar level than the declining blood lactic acid concentration of these same animals at this same time (Griffith, Lockwood and Emery, 1939).

It may be noted that lowering of body temperature is not responsible for this slight decline in resting oxygen consumption. Reference has been made to the fact that special attention was given to maintaining constant rectal temperature; the success of this over the 15–20 minute interval under discussion is evidenced by the fact that the average temperatures of both sets of determinations were exactly equal, 37.7°C.

Inter-individual variability. In spite of this constancy of the resting oxygen consumption in individual animals, inter-individual variability is large: range, 3.7 to 9.3, and average, 6.43 cc. per kilo per minute; standard deviation, 1.23, and coefficient of variation (standard deviation/mean $\times$ 100) 19.2. In this respect, however, oxygen consumption is not unique, but merely resembles the blood sugar and lactic acid concentrations already described, as well as other physiological processes which were measured at the same time. The principal reason for mentioning it here is because it will be shown later that there is a slight tendency for the calorigenic action of adrenalin to be less when the resting metabolic rate is high. It seems improbable, however, that this is responsible in any significant degree for the average results which are to be described since the average resting rates of oxygen utilization for the various groups varied only within a relatively narrow range and were, in cubic centimeters per kilo per minute; with rate of injection in parenthesis: 7.10 (0.00025); 6.11 (0.00050); 5.48 (0.00100); 5.42 (0.00200); 5.96 (0.0040); 7.83 (0.00700); 6.30 (0.01000).

The Effect of Injection. Control injection of isotonic NaCl. Averages of the 10 experiments in which isotonic NaCl was injected in amount and rate similar to that serving as vehicle for adrenalin in the remaining experiments showed values for oxygen consumption, 100, 99 and 101 per cent of normal during the 5-minute injection, and 5 to 10, and 25 to 30 minutes after its completion. In other words the metabolic rate is not only practically constant during the 15 to 20 minute interval preceding injection (previous section) but remains so during a subsequent 35 minutes when isotonic NaCl in this amount is the only substance injected.

These control values have been used as the initial points for the curves of figure 1.

The Effect of Adrenalin Injection. Figure 1 presents the results in two ways: part I includes three curves defining the rate of oxygen consumption during the period of injection (continuous line); and 5 to 10 minutes

(dashed line), and 25 to 30 minutes (dotted line) after injection was completed. The single curve of part II depicts the approximate total change effected by each rate of injection for the entire 35-minute experimental period, as obtained by summing the products of the above values when multiplied by 5, 10 and 20 (minutes), respectively. Though not an exact summation of the area beneath the time curve for each rate of injection it is sufficiently exact for present purposes.

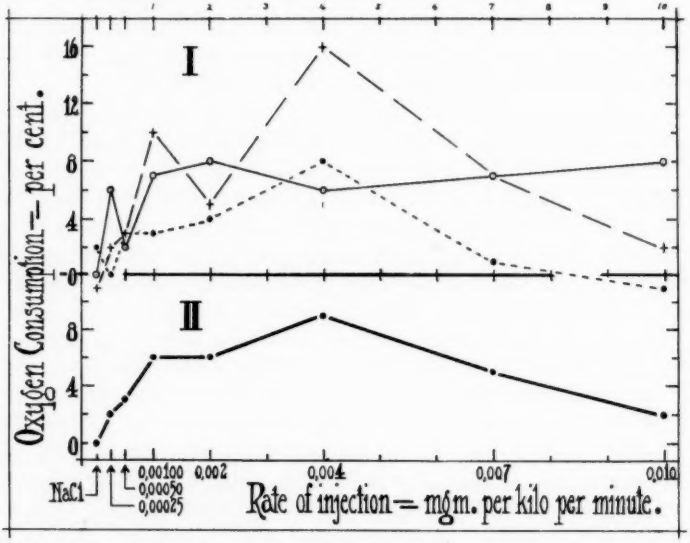

Fig. 1. The effect upon oxygen consumption of intravenous injection of isotonic NaCl solution and of adrenalin at rates of 0.00025, 0.00050, 0.00100, 0.00200, 0.00400, 0.00700 and 0.01000 mgm. per kilo per minute for 5 minutes.

I. The upper three curves: Continuous line: the average value during the 5-minute injection period. Dashed line: values during the interval 5 to 10 minutes after the end of injection. Dotted line: values during the interval 25 to 30 minutes after the injection.

II. Heavy, continuous line: total summated change during the 35-minute period, including the 5-minute injection and the 30 minutes following.

Threshold. It is doubtful from our results whether 0.00025 or 0.00050 mgm. per kilo per minute is the minimal effective rate of intravenous injection required to increase oxygen consumption. The increase during the 5-minute injection period is 6 per cent with the smaller dose and 2 per cent with the larger; with no significant changes in either instance at the 5 to 10 and 25 to 30 minute intervals after injection. It is obvious both of these results cannot be correct; and although there is no internal

evidence as to which is more grossly in error, outside considerations would make it doubtful that the lesser dose could have so large an effect. Thus, in the first of the previous reports of this series, 0.00050 mgm. per kilo per minute was shown to be the threshold for the more accurately determinable hyperglycemia. And although there is nothing *a priori* to prevent the calorigenic effect having a lower threshold than the hyperglycemia, it was found by Cori and Buchwald (1930) in normal men that the reverse was true: 0.000025 mgm. per kilo per minute increasing blood sugar, whereas increased metabolic rate was first detectable only at twice this rate of injection. It seems likely, therefore, that 0.00050 mgm. per kilo per minute is probably nearer the threshold value for the calorigenic effect in these anesthetized cats. Contrary to the findings of Cori and Buchwald, this would make the threshold for hyperglycemia and the metabolic effect equal and at a value one-half that required to produce certain elevation of the blood lactic acid level. It also indicates a higher threshold for these anesthetized cats than for normal unanesthetized men; a decrease in sensitivity which was also found for both blood sugar and blood lactic acid elevation as recorded in the two previous reports of this series.

Proportionality between rate of injection and calorigenic action. Consideration of this relationship will be facilitated by dealing separately with: 1, the results obtained during the 5-minute injection period; 2, the results 5 to 10 and 3, 25 to 30 minutes after injection; and 4, the total summated effect for the entire 35-minute experimental period.

1. *The increased oxygen utilization occurring during injection* (fig. 1, I, continuous line), with the exception of the doubtful and seemingly aberrant result of the lowest rate of injection (as explained in the previous section) the results appear consistent and uniform and indicate that beyond the probable threshold of 0.00050 mgm. per kilo per minute with its 2 per cent elevation of metabolic rate the increase of oxygen consumption occurring during the 5-minute injection period is practically the same for all of the other rates of injection that were used; the increases in per cent, with the rate of injection in parenthesis, being: 7 (0.001); 8 (0.002); 6 (0.004); 7 (0.007); 8 (0.010). This unexpected, all-or-nothing type of response is unlike either the hyperglycemia, which rose to a maximum and then declined; or the increase in blood lactic acid concentration, which was more or less proportional to dose over the whole range of these same injections in these same animals (two previous reports).

This uniformity of average result, indicating that the immediate calorigenic effect is independent of rate of injection, makes it permissible to treat the entire data of this section as a unit for certain general purposes of not too exact a nature. Summarizing, then, the effect during injection

of adrenalin, irrespective of its rate of administration, the following statistics are obtained:

Number of observations	74	
Oxygen consumption, per cent of normal:		
Range	75–133	per cent
Mode	109	per cent
Mean	106	per cent
Standard deviation	10.6	per cent
Coefficient of variation	9.9	per cent

As shown by the range, the effect is highly variable. Breaking this down still further, 19 of the 74 injections were accompanied by sub-normal oxygen consumption; this again is unrelated to rate of injection, the number of sub-normal results at the respective rates (in parenthesis) being: 4 (0.00025), 3 (0.00050), 3 (0.001), 1 (0.002), 4 (0.004), 2 (0.007), 2 (0.010).

In attempting to account for this variability it was thought that although it would not account for the negative results, perhaps a metabolic stimulant, to produce uniform effect, should be administered in proportion to body surface rather than weight; correlation between body weight and percentage change in oxygen consumption which should be appreciable if such a supposition were true is, however, insignificant: $+0.097 \pm 0.079$.

The effect of injecting adrenalin is, however, in individual animals, significantly related to the resting normal metabolic rate; correlation of this with percentage change during injection being -0.435 ± 0.064. It is therefore important that the average resting oxygen consumption, as pointed out in a previous section, was fairly uniform for each of the injection groups and cannot be suspected of having appreciable effect on the average results of the curve of figure 1.

2. *The rate of oxygen utilization 5–10 minutes after injection* (fig. 1, I, dashed line). This curve also has one apparently aberrant point: the effect with 0.002 mgm. per kilo per minute. Disregarding this, the results indicate a rise to a maximum effect (+16 per cent) with injection at the rate of 0.004 mgm. per kilo per minute; and uniform decrease with the two rates of injection greater than this. Unexpected as this result also may be, it is almost identical, qualitatively, with that describing blood sugar concentration 10 minutes after injection in these same animals; but is completely unlike the course of blood lactic acid change at this time (see the two earlier reports).

Also employing the smoothed curve (i.e., disregarding the abnormal irregularity at 0.002 mgm. per kilo per minute) it would appear that at rates of injection between threshold (0.00050) and 0.007 mgm. per kilo per minute, the metabolic effect of adrenalin is not confined merely to its period of injection but persists with ever-increasing effect into the 10-minute interval following. The result of this is that the maximum average

increase of oxygen consumption observed in this work (+16 per cent) occurred during this interval following injection at the rate of 0.004 mgm. per kilo per minute.

This persistence and augmentation of effect following injection was also observed in the simultaneously determined changes in blood sugar and lactic acid concentrations. But, again, the hyperglycemia and calorigenic effect offer the closest parallel: both being maximum after injection at the rate of 0.004 mgm. per kilo per minute and falling off as rate of injection is increased beyond this.

The simplest explanation that suggests itself to account for this persistence of effect beyond the period of actual injection is a destruction of adrenalin less rapidly than is commonly supposed and its retention in the blood or tissues to exercise a continuing effect after injection is over. This, which was mentioned as a possibility in the preceding report on adrenalin hyperglycemia and referred to also in the description of the blood lactic acid effects, was left for final criticism until the evidence here presented was in hand.

Some such theory might account for the mere persistence of effect or a return to normal less rapid than expected. It cannot, however, explain two characteristics especially shown by the hyperglycemia and calorigenic action: 1, within the lower range of doses the effect continues to *increase* during the post-injection period; 2, with the two highest rates of injection this after-effect is increasingly less than with smaller doses. Neither of these would seem compatible with the idea of mere retention of undestroyed injected adrenalin: for presumably this could be no larger than its source; and it would be expected to vary in proportion with the amount injected.

3. *The rate of oxygen utilization 25–30 minutes after injection* (fig. 1, I, dotted line). Following all except threshold rate of injection, metabolic rate shows substantial decline during the 15 to 20 minute interval which separates this terminal determination from its predecessor. In keeping with the diminished immediate after-effect of the two highest rates of injection, oxygen consumption has returned practically to normal at the end of this half-hour period following administration at the rates of 0.007 and 0.010 mgm. per kilo per minute. It is still 3, 3, 4 and 6 per cent above normal following injection at rates of 0.00050, 0.001, 0.002 and 0.004 mgm. per kilo per minute, respectively.

4. *Total, summated calorigenic effect for the entire 35-minute experimental period* (fig. 1, II). The continuous, uniform increase in oxygen consumption which is shown by this curve as rate of injection is increased up to 0.004 mgm. per kilo per minute is similar to the effect previously described for both blood sugar and lactic acid concentrations. Beyond this rate, however, and in keeping with the effects 5 to 10 and 25 to 30 minutes after injection, the total calorigenic action is progressively diminished, parallel-

ing in this respect, also, the hyperglycemic effect, but quite different from the total change in blood lactic acid, which continues to increase in unbroken uniformity with increased rate of injection.

COMMENT. This report makes available for comparison quantitative changes in blood sugar and lactic acid concentration (two previous reports) and oxygen consumption as these are affected by adrenalin in various concentrations from minimally effective to maximally tolerated. When they were begun it was hoped determination of simultaneous variation of these three fundamental metabolic processes would help to elucidate their possible interrelationships and mechanism of control by adrenalin. The issue was not prejudged; but at the time, direct relationship between the hyperglycemia of adrenalin and its calorigenic effect seemed definitely disproved, at least in so far as concerned the possibility of the increased metabolic rate being merely a consequence of the experimentally induced carbohydrate plethora (Boothby and Sandiford, 1923); whereas the calorigenic action seemed merely a phase of lactic acid metabolism as a result of the beautiful quantitative work of the Coris (Cori, 1931).

The essential conclusion from the work of Boothby and Sandiford was that adrenalin owed its calorigenic action to direct cellular stimulation which they and, later, Hunt and Bright (1926) envisaged as occurring throughout the body generally but particularly in muscle as the largest mass of active tissue. In this respect the Coris might be regarded as merely implementing this general theory by identifying the stimulatory process with muscle glycogenolysis and the metabolic disposal of the released lactic acid. If the calorigenic effect and the mobilization of glucose are independent there would of course be no necessary connection between the increase in oxygen consumption and increase in blood sugar level although each might show a definite proportionality with adrenalin dosage. Nor, in view of the fact that blood concentration represents merely the balance between entry and exit, would there of necessity be any relationship between calorigenic action and blood lactic acid concentration, although identification of calorigenic action with lactic acid formation and disposal might lead to tentative expectation of a more definite relationship than with the, presumably, completely independent process of blood sugar mobilization.

Our results must be taken as purely descriptive and without causal significance; nevertheless when considered in relation with other recently completed work, they only make more, rather than less difficult, complete identification of the calorigenic action of adrenalin with lactic acid metabolism, especially in skeletal muscle. Thus it has been shown: 1, that increases of blood lactic acid occurring during splanchnic-adrenal stimulation may be almost entirely accounted for by visceral constriction (Griffith and Emery, 1935); 2, after evisceration, although the entire skeletal

musculature is intact and served by adequate blood pressure, blood lactic acid not only fails to increase normally but usually suffers temporary slight decline during injection of adrenalin (Griffith, Lockwood and Emery, 1938); and finally, 3, it has been impossible to substantiate claims that have been made that adrenalin increases the metabolic rate of isolated muscle (Griffith, 1923) or of peripheral tissues when supplied to them by inter-arterial injection (Cammer and Griffith, 1939).

On the other hand, for what it may be worth, there is remarkably (and it must not be overlooked, perhaps, deceptively) close parallelism between increase in blood sugar concentration and increased oxygen consumption in response to various concentrations of injected adrenalin: thus both have the same threshold; both rise to maximum in the immediate post-injection period after administration at the rate of 0.004 mgm. per kilo per minute; and both are increased less as rate of injection exceeds this.

This last, the decrease in effect as rate of injection exceeds 0.004 mgm. per kilo per minute, is warning, if any were needed, that even the closely parallel effects on carbohydrate mobilization and oxygen utilization cannot be regarded as uncomplicated results of direct cellular action. Nor can the diminished hyperglycemia following high rates of administration of adrenalin be attributed to increased oxidative utilization of blood sugar, since the total metabolic rate decreases in proportion. Undoubtedly vascular effects which assume increasing importance as dosage increases, by redistribution of blood and modification of the access of adrenalin to and escape of glucose or lactic acid from various parts of the body, must necessarily obscure direct effects on cellular metabolism. This makes it all the more surprising that changes of oxygen consumption show such close parallelism with changes of blood sugar concentration and so little relation with blood lactic acid level.

SUMMARY

Intravenous injection of adrenalin at rates of 0.00025, 0.00050, 0.00100, 0.00200, 0.00400, 0.00700 and 0.01000 mgm. per kilo per minute for five minutes affects the oxygen consumption of chloralose-anesthetized cats as follows:

1. The minimal effective rate of injection is either 0.00025 or 0.00050 mgm. per kilo per minute and more probably the latter.

2. At all rates of injection above threshold, oxygen intake is increased approximately the same amount (+6 to +8 per cent) during the 5-minute injection period itself.

3. At rates of injection below 0.007 mgm. per kilo per minute oxygen consumption continues to increase to a maximum 5 to 10 minutes after injection is over; the maximum calorigenic effect (+16 per cent) occurs at this time after injection at the rate of 0.004 mgm. per kilo per minute.

4. At the two highest rates of injection, 0.007 and 0.010 mgm. per kilo per minute, oxygen consumption during injection is increased no more and no less than with smaller rates of administration; the total effect following injection is, however, less.

5. The total calorigenic effect for the entire 35-minute experimental period increases from threshold to maximum at 0.004 mgm. per kilo per minute and then diminishes progressively at rates of injection beyond this.

6. The calorigenic effect parallels very closely in threshold, maximum and decline at higher rates of injection, the simultaneous changes in blood sugar level but is quite unlike the increases in blood lactic acid.

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SUCROSE TASTE THRESHOLDS OF RATS AND HUMANS

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In a previous paper it was reported that rats and humans have almost the same taste threshold for sodium chloride (Richter and MacLean, 1939). Normal rats distinguished salt solutions from distilled water in an average concentration of 0.058 per cent, while humans first recognized the taste of salt in solutions in an average concentration of 0.065 per cent.

We were interested to know if rats and humans have the same thresholds for other substances. The following experiments deal with the taste thresholds for sucrose.

METHODS. The techniques used with rats and humans have been described in detail in previous papers (Richter, 1939; Richter and MacLean, 1939).

We kept the rats separately in cages equipped with a food cup (standard McCollum diet) and two graduated inverted drinking fountains. The room temperature was maintained at 75 to 78°F. During the first few days of the experiment we filled both bottles with distilled water. In most cases, after 10 to 15 days, the intake from each bottle reached a fairly constant level. At this time we filled the bottle in the right corner of the cage with sucrose solution in a subliminal concentration, and thereafter increased the concentration daily in small steps from 0.01 to 1.60 per cent, or from 0.0003 to 0.047 mol. See table 1.

In order to keep conditions constant, we used the same two bottles for each rat throughout the experiment. Further, we placed each substance consistently in the same bottle and in the same corner of the cage—the distilled water on the left, the sucrose solution on the right.

All glassware was cleaned in sulphuric acid-potassium dichromate cleaning solution and thoroughly rinsed in tap water followed by distilled water.

As in the salt taste experiments, it was found that when the concentration reached a certain point the rats began to drink more sucrose solution and less distilled water, an indication that they distinguished between the two. This point was taken to be the sucrose taste threshold.

Thirteen young adult rats were used in these experiments.

Essentially the same technique was used on human subjects. The blindfolded subjects were comfortably seated opposite the experimenter at a small table. Two 1-ounce glasses filled at approximately 10 cc.—one with distilled water, the other with a sucrose solution—were put into the subject's hands, and instructions were given to compare the taste of the two liquids by sipping from the glasses and to describe the sensations derived from each one. The subjects were allowed to taste first one solution then the other several times until they felt that they had given a correct description of their sensations. The concentrations were increased in exactly the same order used in the experiments with the rats. The glasses were interchanged in an irregular order so that at one time the subject received the sugar solution in the right hand and at another time in the left hand. The experiments were continued until the subjects first stated that they recognized a difference between the sugar solution and the

TABLE 1

Concentrations of sucrose in per cent

0.01	0.30	1.00
0.02	0.40	1.10
0.04	0.50	1.20
0.06	0.60	1.30
0.08	0.70	1.40
0.10	0.80	1.50
0.20	0.90	1.60

distilled water and until they finally stated that they recognized a sweet taste. The former was taken as the difference threshold; the latter, as the sucrose taste threshold.

In our previous experiments we tested the subjects with each of the solutions, not skipping any of them, starting with the lowest and stopping with the one which gave the recognized taste. This meant that usually the subjects had to drink a large amount of water, as much as 400 cc. in some instances. In order to eliminate this difficulty we increased the concentrations in large steps, skipping one or two solutions each time, until we reached a point at which the subjects recognized a difference between the two solutions. Then we decreased the concentration in small steps, until we determined the lowest concentration at which the subjects could still detect a difference. This point was taken as the taste difference threshold. We used the same procedure to determine the sucrose taste threshold.

We kept a record of the drinking and food habits of each subject, also a record of the condition of the teeth, gums, and tongue.

The sucrose solutions were made fresh daily in volumetric flasks. Distilled water used in the comparison glass and also in the sucrose solutions

was taken from the same source, thereby keeping the temperature constant for both glasses. The temperature over a period of days was found to vary from 19 to 29°C. The tests were taken between 10 a.m. to 12 a.m. and 1:30 p.m. to 4 p.m. At least one hour was allowed after meals. The majority of subjects did not know the solutions contained sugar.

Because of inconsistent descriptions of taste sensations an occasional subject was thought to be guessing. Questionable reports were checked by repeating the same concentrations of sucrose solution, or by giving distilled water in both glasses.

Threshold determinations were made on 174 subjects: 19 adults (19–50 years of age) from the laboratory staff and clinic patients; 45 adults (19–21 years) and 58 children (7–10 years) from the State Teachers College at Towson, Maryland; and 52 individuals (52–85 years) from the Old Age Infirmary of the Baltimore City Hospital.[1]

RESULTS. *Sucrose taste thresholds of rats.* Figure 1A shows a typical record of one of the 13 rats. The ordinates give fluid intake in cubic centimeters; the abscissae, time in days and concentrations of the sucrose solution. During the preliminary period of 17 days in which both bottles contained distilled water, the intake of each bottle fluctuated considerably, but each averaged approximately 12 cc. per day. The arrow indicates the day on which we started filling one bottle with a sucrose solution and increasing the concentration each day according to table 1. For 8 days the intake from the two bottles still remained approximately the same. On the 9th day, however, when the concentration reached 0.4 per cent, the intake of the sucrose solution increased; and the intake of distilled water decreased. Thereafter the intake of sucrose solution increased steadily while the intake of distilled water decreased.

Figure 1B summarizes the results for the 13 rats. The concentration of sucrose solution at which the rats first definitely indicated that they recognized a difference fell at 0.5 per cent with a variation from 0.3 to 0.8 per cent.

Sucrose taste thresholds of humans. The 45 adults at the State Teachers College first distinguished the sucrose solution from distilled water at concentrations which averaged 0.41 per cent (weighted for frequency). Figure 2A and 2B give the frequency curves for the taste difference and sweet taste thresholds of this group. The taste difference thresholds varied from 0.01 to 0.5 per cent; the sweet taste thresholds, from 0.1 to 1.0 per cent. The 19 adults from the hospital and laboratory staff had essentially

[1] For their kind coöperation in these experiments we wish to express our gratitude to Dr. M. Theresa Wiedefeld, President, and Miss Catherine N. Cook of the State Teachers College at Towson, Maryland, and to Dr. P. J. McMillin, Superintendent, and Mr. Richard R. Griffith, Infirmary Superintendent, of the Baltimore City Hospital.

the same thresholds. The 58 children from the training school of the Teachers College gave very similar results. However, their thresholds averaged slightly higher, 0.44 per cent for the difference threshold and 0.68 per cent for the sweet taste threshold. In addition, 4 children did not recognize the sweet taste until the concentration reached 2 per cent, and 2 children failed to recognize the sweet taste even with a 10 per cent solution. Attention difficulties may possibly explain some of the high thresholds.

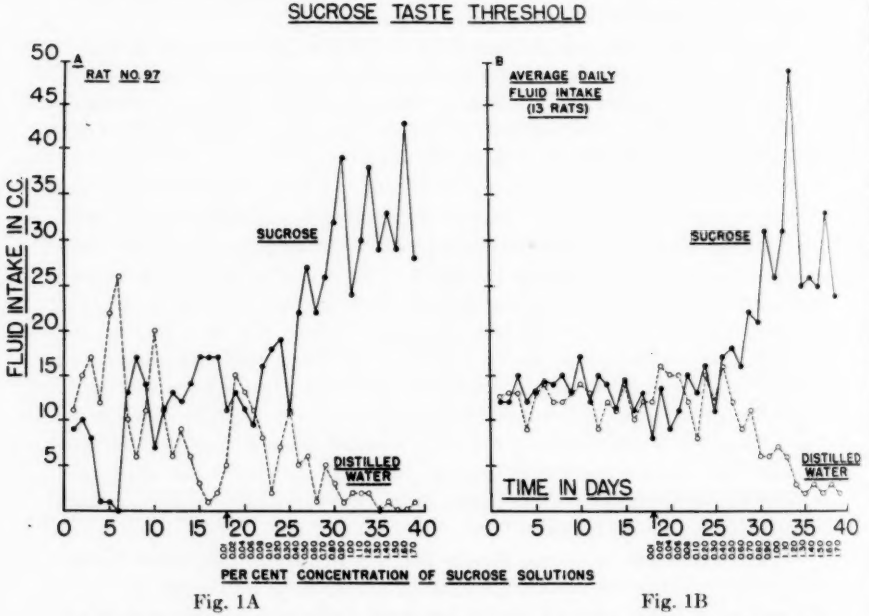

Fig. 1A Fig. 1B

Fig. 1A. Daily intake curves of distilled water and sucrose solutions showing the per cent concentration at which a typical rat manifested a preference for sucrose. The arrow indicates the day on which the sucrose solution replaced the distilled water in the second bottle.

Fig. 1B. Average daily intake curves of distilled water and sucrose solutions showing the average per cent concentration at which 13 rats manifested a preference for sucrose.

The 52 elderly individuals from the Old Age Infirmary of the Baltimore City Hospital gave an even higher average and high individual thresholds. Since most of these subjects did not distinguish between the distilled water and the sucrose solution until they recognized the sweet taste, their taste difference and sweet taste thresholds coincided at 1.23 per cent and ranged from 0.01 per cent to more than 5 per cent. Eleven subjects did not recognize the sweet taste until the concentration reached 2 per cent; five recognized it at 3 per cent; and one, at 4 per cent. Two failed to recognize

the sweet taste even at 5 per cent. The greater atrophy of the papillae and taste buds in elderly individuals might explain their higher taste thresholds.

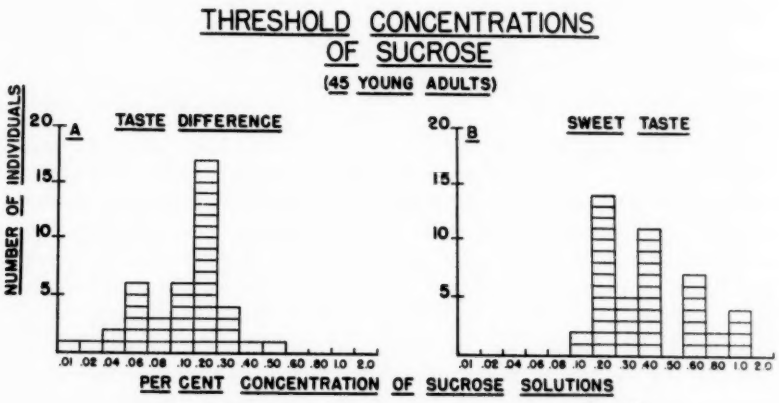

Fig. 2A Fig. 2B

Fig. 2A. Frequency curve showing the per cent concentrations of sucrose solutions at which 45 young adult humans first distinguished a taste difference between a sucrose solution and distilled water.

Fig. 2B. Frequency curve showing the per cent concentration of sucrose solution at which the same 45 young adult humans first recognized a sweet taste.

TABLE 2

Expressions used to describe subliminal sucrose solutions and frequency of use

TASTE SENSATION	NUMBER OF SUBJECTS	TASTE SENSATION	NUMBER OF SUBJECTS
Bitter	12	Musty	2
Sour	8	Iron	2
Salty	7	Mineral	2
Acid	6	Ether	1
Chemical	6	Chalky	1
Medicine	5	Alcohol	1
Lemon	4	Wine	1
Peppermint	3		

REMARKS. In concentrations below the sweet taste threshold, the sucrose solution elicited a variety of taste sensations, most often bitter. Table 2 summarizes the results.

DISCUSSION. The results of these experiments show that rats and humans have very similar sucrose taste thresholds, just as they do salt taste thresholds.

These taste threshold reports agree closely with most of those reported previously by other workers, a few of which appear in the following list:

Venable	1887	0.3 per cent
Bailey and Nichols	1887	0.5 per cent
Hänig	1901	0.35 per cent
Haemelinck	1905	0.4 per cent
Lemberger	1908	0.44 per cent
Blakeslee and Salmon	1935	1.28 per cent (maximum frequency)

Henning's review on taste (1921) contains a fuller list. We have quoted only those references which we were able to verify.

The following conditions were not found to affect taste sensitivity for sucrose: chronic alcoholism of 25 years' duration, excessive smoking of cigarettes, badly infected gums, marked decay of teeth, mild head colds, hay fever, or allergy. Coating of the surface of the tongue apparently had little influence, but there was some evidence that degeneration of the papillae at the margins of the tongue, as indicated by smoothness, was correlated with less sensitivity of taste for sucrose. However, more study is necessary to establish this correlation. Data are insufficient to determine if dislike of sugar affected taste sensitivity of sucrose; but loss of appetite for food, in general, apparently had no effect.

The fact that some individuals did not recognize a sweet taste even when concentrations as high as 10 per cent were offered may indicate the presence of a taste blindness for sucrose. The existence of such high thresholds may help to explain the failure of some individuals to make beneficial dietary selections on the basis of taste.

Within the range of temperatures of the solutions used in these experiments the taste thresholds varied only slightly, if at all. This does not agree with the reports of Hahn, Kuckulies and Taeger (1938), who found a wide degree of variation of sucrose taste thresholds with relationship to temperature of the solutions. This discrepancy may be explained, at least in part, by the fact that they worked with solutions which had a wider range of variation, 17 to 31°C., and also they tested only a small area of the tongue and with a method which excluded the saliva and did not allow the solution to take on the temperature of the body. With our method the small amount of solution held in the mouth must quickly have become mixed with the saliva and have taken on the temperature of the mouth.

SUMMARY

1. Thirteen rats recognized the difference between distilled water and sucrose solution in average concentrations of 0.5 per cent (or 0.0146 mol.).

2. Forty-five young adult humans first recognized the difference between distilled water and sucrose solution in average concentrations of 0.17

per cent (or 0.005 mol.) and first recognized a sweet taste in average concentrations of 0.41 per cent (or 0.012 mol.). Nineteen adults from the laboratory staff and clinic patients showed similar results.

3. The sugar taste threshold of 58 children averaged 0.68 per cent and of 52 elderly individuals, 1.23 per cent.

4. Since the sucrose taste threshold of the rats (0.5 per cent) agreed more closely with the sweet taste threshold of humans (0.4 to 0.7 per cent) than with the taste difference threshold (0.17 per cent), it would seem likely that the rats did not start to drink more sucrose and less water until they recognized a definitely sweet taste.

5. It was concluded that rats and humans have nearly the same sucrose taste thresholds.

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THE EFFECT OF ACID STIMULATION OF THE DUODENUM UPON EXPERIMENTAL HYPERGLYCEMIA AND UTILIZATION OF GLUCOSE

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The concept of a duodenal hormone which aids in the regulation of carbohydrate metabolism has been based on reports that the blood sugar concentration is reduced following *a*, injection or ingestion of certain extracts of the intestinal mucosa, and *b*, stimulation of the duodenal mucosa with dilute hydrochloric acid. We have recently published data (1) which show that the introduction of dilute hydrochloric acid into the duodenum of dogs does not reduce the *fasting* blood sugar concentration. It is possible, however, that the proposed hormone mechanism may be effective in reducing experimentally induced hyperglycemia. Since the literature is not conclusive on this point (2–6), studies were made upon dogs to determine the effect of acid stimulation of the duodenum upon *a*, alimentary hyperglycemia; *b*, utilization of intravenous glucose; *c*, adrenalin hyperglycemia, and *d*, hyperglycemia of depancreatized dogs.

METHODS. In order to permit injections of acid or saline into the duodenum of chronic animals a small rubber catheter was inserted into the duodenum at an operation performed aseptically under anesthesia and allowed to protrude through the abdominal wall. At the same operation Thiry-Vella ileal loops, three to five feet in length, were prepared in three dogs. The loops were fitted with chrome-plated metal cannulae to facilitate the introduction, retention, and removal of glucose solutions. These ileal loops were irrigated with saline solution almost daily during the time required for the animals to recover from the operation, and later, upon days when experiments were not conducted. Experimentation was not begun upon chronic animals until they were in good physical condition ten days to three weeks following the operation. During this period the animals were trained to stand in stocks or to lie upon a padded bed for several hours. The dogs were deprived of food 12 to 24 hours before the experiments were performed.

The method of Schaffer, Hartman and Somogyi (reagent no. 2) (7) was

[1] Porter Fellow in Physiology, 1938–39.

employed in making blood sugar determinations upon Somogyi's zinc hydroxide blood filtrates (8) representing 2.0 cc. of venous blood. The intestinal washings secured in the experiments dealing with alimentary hyperglycemia in anesthetized and unanesthetized dogs were properly diluted and treated the same as blood samples in the analyses made to determine the concentration of glucose. The intestinal loops were always washed with saline until the washings were free of sugar. In the glucose utilization experiments, urinary sugar was determined by Benedict's quantitative method.

RESULTS. A. *Alimentary Hyperglycemia. Anesthetized dogs.* Following anesthetization with pentobarbital, and laparotomy, two intestinal segments were formed by placing a ligature about the jejunum approximately twelve inches below the ligament of Treitz, and a third ligature about the ileum immediately above the cecum. A large hypodermic needle inserted into the proximal end of the lower intestinal segment and connected to rubber tubing permitted the introduction of a 10 per cent solution of freshly prepared glucose (12 cc. per kgm. body weight) in 5 to 8 minutes. In the same manner, injections of 50 cc. of 0.3 per cent hydrochloric acid or, in the control experiments, the same quantity of 0.9 per cent saline, were made into the duodenum during the first 20 minutes of the experimental period.

The average hyperglycemic level induced in the 17 animals in which the duodenum was stimulated with acid was lower than that induced in the 21 control animals (table 1). The difference, which was greatest at the end of one hour, amounted to 16 mgm. per cent. However, an examination of the blood sugar data revealed a wide variation between individual dogs and an extensive overlapping of the curves obtained from the test and control experiments. This extreme variability in response is illustrated by representative experiments included in table 1.

A statistical analysis indicates that the differences in the average curves are not significant.

When it first became apparent that wide variations in glycemic curves occurred, attempts were made to eliminate them by maintaining the animals for at least a week on a standard diet. The variations persisted however, and furthermore, could not be related to variations in the quantity of glucose absorbed. Trimble, Carey and Maddock (9) have previously commented on these marked variations in alimentary hyperglycemic curves in unanesthetized dogs. Because of these uncontrollable variations, we attach no significance to the results obtained in this type of experiment.

Unanesthetized dogs. It was deemed advisable to make further studies upon the glucose tolerance during acid stimulation of the duodenum in unanesthetized dogs provided with Thiry-Vella loops in order that the

same animals could be used repeatedly for both test and control experiments. The glucose solution was introduced into the ileal loop with slight pressure over a period of 3 to 5 minutes. During the first 20 minutes of the one-hour absorption period, 50 cc. of 0.3 per cent hydrochloric acid were introduced into the duodenum. The glucose solution was drained out after one hour, and the loop was thoroughly washed with warm saline solution. The experimental procedure as outlined was repeated upon the

TABLE 1

The effect of duodenal stimulation with dilute hydrochloric acid upon the alimentary hyperglycemia of anesthetized dogs

Dogs 10, 4, 5 and 11 illustrate the extremes in variability.

TREATED GROUP		BLOOD-SUGAR CONCENTRATION (MGM. PER CENT)								GLUCOSE IN LOOP	GLUCOSE OUT	GLUCOSE ABSORBED	GLUCOSE ABSORBED/KGM./2.5 HRS.
		Control period		50 cc. 0.3 per cent HCl in duodenum, 12 cc. 10.0 per cent glucose/kgm. in loop									
Dog number	Weight	0.5 hr.	1.0 hr.	20 min.	40 min.	60 min.	90 min.	120 min.	150 min.				
	kgm.									*grams*	*grams*	*grams*	*grams*
10	8.40	69	71	85	77	75	72	67	64				
4	10.20	100	101	153	174	185	142	104	92				
Average (17 dogs)	11.28	84	83	103	111	113	107	97	93	13.19*	2.50*	10.69*	0.98*
PE_m		±1.54	±1.56	±3.44	±4.13	±4.67	±4.13	±3.88	±3.63				
CONTROL GROUP				50 cc. 0.9 per cent NaCl in duodenum, 12 cc. 10.0 per cent glucose/kgm. in loop									
5	19.30	84	78	87	90	85	84	84	86				
11	13.60	93	92	129	163	176	174	153	159				
Average (21 dogs)	11.74	82	83	109	124	129	118	107	93	13.39†	1.67†	11.72†	1.04†
PE_m		±1.61	±1.57	±2.89	±3.98	±4.41	±4.52	±3.84	±2.90				
$(M_1 - M_2) \pm PE$				6± 4.49	13± 5.73	16± 6.42	11± 6.12	10± 5.46	0± 4.64				

* Average of eleven dogs.
† Average of nine dogs.

following day with 50 cc. of 0.9 per cent saline being substituted for the acid placed in the duodenum.

The average blood sugar concentration during the absorption of glucose for one hour and for a one-hour post-absorptive period was determined in 22 experiments upon 3 dogs. In one-half of these experiments dilute acid was instilled into the duodenum but the average blood sugar concentration was practically identical to that found during the same number of control experiments (table 2). Thus, the data secured during the course of these

experiments upon *non-anesthetized* dogs do not even suggest that acid stimulation of the duodenum improves the glucose tolerance. These data also support the view that the apparent improvement in glucose tolerance of the *anesthetized* dogs when acid was placed in the duodenum should be attributed to chance variation.

TABLE 2

The effect of acid stimulation of the duodenum upon the blood sugar concentration following glucose absorption from Thiry-Vella loops

DOG NUMBER	EXPERIMENT NUMBER	WEIGHT	FAST	BLOOD SUGAR CONCENTRATION (MGM. PER CENT)							GLUCOSE IN LOOP		AMOUNT OF GLUCOSE ABSORBED	
				Fasting blood sugar	50 cc. 0.3 per cent HCl in duodenum									
					Absorption period			Post-absorption period						
					20 min.	40 min.	60 min.	80 min.	100 min.	120 min.	Grams	Per cent	Grams	Gm./kgm./1 hr.
		kgm.	*hours*											
1 ♂	1	13.60	20	56	69	88	92	85	71	61	18.00	7.5	5.16	0.378
2 ♂	2	15.91	20	61	61	67	77	63	56	60	18.00	7.5	7.76	0.487
	3	15.45	15	69	91	87	91	74	63	69	18.00	7.5	11.40	0.738
	4	15.91	13	65	91	75	75	73	62	70	18.00	7.5	4.84	0.304
3 ♀	5	18.52	14	70	77	79	79	73	73	76	18.00	7.5	6.00	0.324
2 ♂	6	14.50	17	76	84	80	84	73	64	76	21.60	13.5	5.00	0.344
1 ♂	7	12.73	19	52	81	107	115	97	75	63	28.48	13.5	8.64	0.680
	8	11.60	19	58	68	92	95	90	68	64	23.49	13.5	6.25	0.539
	9	11.80	20	69	94	119	108	96	77	61	23.89	13.5	8.35	0.707
2 ♂	10	15.23	19	73	77	85	85	65	65	63	24.00	10.0	14.35	0.942
	11	15.45	20	69	71	85	90	65	60	59	24.00	10.0	7.36	0.476
Average ...		14.61	17	64	78	87	90	77	66	65			7.74	0.538
					50 cc. 0.9 per cent NaCl in duodenum									
1 ♂	1	13.60	20	58	79	89	90	74	56	52	18.00	7.5	7.08	0.520
2 ♂	2	15.23	20	62	73	75	84	71	60	60	18.00	7.5	8.40	0.558
	3	15.45	17	63	91	94	96	75	61	59	18.00	7.5	9.64	0.637
	4	16.14	12	61	72	74	87	59	61	58	18.00	7.5	10.80	0.669
3 ♀	5	18.63	15	75	74	74	76	74	72	75	18.00	7.5	5.76	0.309
2 ♂	6	15.00	16	70	92	93	85	72	72	66	21.60	13.5	7.75	0.516
1 ♂	7	12.50	20	53	75	102	111	91	76	64	25.31	13.5	8.49	0.679
	8	12.04	16	57	84	111	105	92	85	75	24.30	13.5	7.66	0.636
	9	11.80	20	56	73	107	118	91	71	58	23.89	13.5	9.57	0.811
2 ♂	10	15.91	16	66	73	74	91	71	64	64	24.00	10.0	10.20	0.641
	11	15.20	19	61	73	79	78	63	56	63	24.00	10.0	9.46	0.822
Average.....		14.68	17	62	78	88	92	75	67	63			8.64	0.599

The data depicting the absorption of glucose from the Thiry-Vella loops (table 2) reveal the interesting fact that the animals absorbed slightly *less* glucose when acid came in contact with the duodenal mucosa than when saline replaced the acid. Kokas and Ludany (10) have recently reported that acid stimulation of the duodenum liberates a hormonal sub-

stance, designated as "villikinine," which augments the intestinal absorption of glucose by 20 per cent by stimulating the movements of the intestinal villi. The physiological significance of this observation must remain in question, especially in view of the fact that the ileal loops of our animals, measuring 44 to 68 inches in length, presented a large area of intestinal mucosa to the glucose which was administered.

B. *Glucose Utilization.* The failure to demonstrate that acid stimulation of the duodenum tends to diminish the hyperglycemia during alimentary absorption of glucose may have been due to the fact that the glucose itself, when in contact with the intestinal mucosa of the acid-treated and saline-control animals, liberates a hormone. In order to investigate this possibility and the additional possibility that the duodenal influence is only demonstrable at very high blood sugar levels, it was deemed necessary to determine whether acid stimulation of the duodenum enhances the utilization of glucose administered *intravenously.*

The ability of the animal organism to utilize glucose was determined by making a continuous intravenous injection of 2.0 grams of glucose per kilogram body weight at a constant rate over a period of one hour. The amount of glucose utilized, expressed in terms of gm./kgm./1 hr., was determined by subtracting the glucose excreted in the urine from the total amount injected. The procedure employed was essentially that first described in detail by Woodyatt *et al.* (11) and used by de Takáts and Cuthbert (12, 13), Wierzuchowski (14) and others. "Pfanstiehl" anhydrous dextrose, in 20 per cent solution, maintained at approximately body temperature, was supplied to the Woodyatt pump from a graduated burette which facilitated an accurate check upon the injection from minute to minute during the one-hour injection period. The animals employed were permitted to urinate before being taken into the laboratory and the urinary bladder was catheterized approximately 30 minutes before the experiment proper was begun. Urine was collected during the injection of glucose and thereafter until the urine gave a negative Benedict's qualitative test for sugar.

The amount of glucose utilized by 2 male and 2 female dogs in 8 experiments during acid stimulation of the duodenum (table 3) was 1.86 gm./kgm./1 hr.; no greater than the amount utilized by the same animals upon the following day when saline was introduced into the duodenum, 1.90 gm./kgm./1 hr. The utilization of glucose by individual animals in both groups of experiments ranged from 1.81 to 1.96 gm./kgm./1 hr., amounts which are within the normal limits ascertained by de Takáts and Cuthbert (12) from a group of fifty dogs, and which compare favorably with the hourly averages determined by Wierzuchowski (14) in six-hour experiments.

It is quite apparent that acid stimulation of the duodenum did not

enhance the utilization of glucose under the conditions of our experiments.

C. *Adrenalin Hyperglycemia.* It has previously been mentioned that in the experiments dealing with alimentary hyperglycemia the glucose itself, in contact with the intestinal mucosa, may have liberated a hormone which obscured any hormonal influence initiated by acid stimulation of the duodenal mucosa. This criticism was obviated, in part, at least, by the experiments which demonstrated that acid stimulation of the duodenum did not improve the efficiency of glucose utilization when liberal amounts of glucose were administered intravenously. It is possible, however, that

TABLE 3

Utilization of intravenous glucose injected at the rate of 2.0 gm./kgm. for one hour

DUODENAL STIMULUS	DOG NUMBER	FAST	WEIGHT	DEXTROSE IN URINE	DEXTROSE ASSIMILATED
		hours	*kgm.*	*gm./kgm.*	*gm./kgm./1 hr.*
50.0 cc. 0.9 per cent NaCl	1 ♂	20	13.41	0.129	1.871
		19	13.64	0.082	1.918
	2 ♂	20	13.63	0.032	1.968
		21	13.66	0.154	1.846
	3 ♀	19	18.82	0.075	1.925
		20	18.98	0.090	1.910
	4 ♀	22	10.00	0.133	1.867
		22	9.54	0.048	1.952
Average					1.907
50.0 cc. 0.3 per cent HCl	1 ♂	20	13.52	0.120	1.880
		21	13.64	0.146	1.854
	2 ♂	21	13.52	0.188	1.812
		24	13.67	0.095	1.905
	3 ♀	20	18.86	0.083	1.917
		20	19.20	0.093	1.907
	4 ♀	22	10.23	0.189	1.811
		22	10.00	0.137	1.863
Average					1.868

a hypoglycemic influence initiated by acid stimulation of the duodenum could be demonstrated during the hyperglycemia induced by injection of adrenalin or by extirpation of the pancreas.

Hyperglycemia was induced in trained, unanesthetized dogs maintained under standard conditions in regard to diet and length of fast by injecting 0.35 mgm. of adrenalin intravenously. Following the injection of adrenalin, blood samples were secured at frequent intervals over a two-hour period. Acid stimulation of the duodenum was employed in 9 experiments, 3 upon each of 3 dogs, and the blood sugar data obtained was compared with that procured from the same number of control experiments

carried out upon the same animals the day following when saline was introduced into the duodenum in place of the acid (table 4). The average blood sugar concentration during acid stimulation of the duodenum was nearly identical to that exhibited by the animals in the control experiments. These consistent results definitely indicate that the magnitude of hyperglycemia following adrenalin injections remained uninfluenced by acid stimulation of the duodenum.

TABLE 4

*The effect of duodenal instillation of HCl upon adrenalin hyperglycemia in unanesthetized dogs**

DOG NUMBER	WEIGHT	FAST	BLOOD SUGAR CONCENTRATION (MGM. PER CENT)						
			Control	50.0 cc. 0.3 per cent HCl in duodenum					
				10 min.	20 min.	30 min.	50 min.	80 min.	120 min.
	kgm.	*hours*							
1 ♂	12.3	17	60	91	84	81	82	68	71
	12.5	18	63	90	87	87	76	66	68
	13.4	20	71	98	83	80	75	68	64
2 ♂	13.2	24	64	80	75	63	63		68
	13.2	22	77	100	96	89	88	75	85
	13.3	21	75	91	89	81	86	80	80
3 ♂	11.6	24	60	92	88	74	64	63	69
	13.2	20	66	99	87	75	65	73	69
	13.4	20	73	99	81	75	79	72	74
Average			68	93	85	78	75	70	72
				50.0 cc. 0.9 per cent NaCl in duodenum					
1 ♂	12.3	18	66	100	96	78	78	71	61
	12.5	21	66	92	81	78	83	69	65
	13.4	21	62	90	95	86	78	66	62
2 ♂	13.2	20	59	78	61	64	60	71	64
	13.2	20	74	87	73	70	74	71	72
	13.3	21	68	79	75	74	74	73	
3 ♂	11.6	22	61	96	72	68	64	66	
	13.2	18	75	103	98	80	77	73	78
	13.4	20	75	91	85	79	81	80	83
Average			67	91	82	75	74	71	69

* Each animal received 0.35 mgm. adrenalin intravenously.

D. *Hyperglycemia of Depancreatized Dogs.* Total pancreatectomy was performed upon a series of 14 dogs. Three days post-operatively the dogs were nembutalized and a cannula was inserted in the duodenum to permit the injections of acid or saline into the duodenum during the first 20 minutes of the experimental period.

The average blood sugar concentration of the 7 depancreatized dogs (table 5) which received 50 cc. of 0.3 per cent hydrochloric acid in the duodenum was not appreciably reduced at any time during the five-hour experimental period. Similar results were obtained from the 7 control animals which received 50 cc. of saline in the duodenum instead of the acid. The averages in each group reveal that a slight increase in blood sugar during the latter part of the five-hour period occurred. This tend-

TABLE 5

Acid stimulation of the duodenum in depancreatized dogs

DOG NUMBER	WEIGHT	BLOOD SUGAR CONCENTRATION (MGM. PER CENT)							
		Control period		50 cc. 0.3 per cent HCl in duodenum					
		0.5 hr.	1.0 hr.	0.5 hr.	1.0 hr.	2.0 hr.	3.0 hr.	4.0 hr.	5.0 hr.
	kgm.								
1 ♀	13.2		227	222	234	247	282	322	355
2 ♂	11.0	253	250	248	251	263	267	262	265
3 ♂	12.7	291	271	263	262	260	256	265	258
4 ♂	14.0	197	195	195	199	204	196	202	208
5 ♂*	11.8	255	244	239	237	242	247	261	249
6 ♀*	12.5	233	246	222	237	216	207	213	210
7 ♀*	12.7	283	283	292	293	311	324	342	380
Average....	12.5	252†	245	240	245	249	268	267	275
				50 cc. 0.9 per cent NaCl in duodenum					
1 ♂	10.4	290	295	292	296	296	297	303	304
2 ♂	14.0	243	239	225	226	221	220	224	222
3 ♂	10.2	233	255	245	265	261	280	295	321
4 ♂	13.8	266	255	253	258	245	246	253	255
5 ♂*	10.2	226	215	226	220	216	210	211	228
6 ♂*	9.4	438	425	435	433	460	493	503	
7 ♂*	10.4	250	255	243	246	228	226	235	243
Average....	11.2	278	277	274	278	275	262	289	262

* Insulin given first and second days post-operatively.

† Average of six.

ency was especially noticeable in 2 animals of each group and resembled the hyperglycemic trend which occurred in the 2 dogs receiving saline in the duodenum which served as controls in similar experiments reported by LaBarre and Ledrut (3). However, none of our animals exhibited an appreciable reduction in blood sugar after acid stimulation of the duodenum.

Discussion. The data secured from carefully controlled experiments designed to simulate normal physiological conditions whenever possible

have consistently failed to yield evidence that acid stimulation of the duodenum reduces the hyperglycemia produced by absorption of glucose, adrenalin injections or removal of the pancreas. Such acid stimulation also failed to enhance the utilization of glucose when liberal amounts were infused intravenously at a constant rate.

The results from several types of experiments reported in the literature have been interpreted to demonstrate that the duodenum has an influence upon the tolerance to alimentary glucose. LaBarre and Houssa (2) believed that the elevated and prolonged glycemic curves obtained from 8 chloralosed dogs following intestinal administration of glucose was due to the possibility that a hypoglycemic substance normally present in the duodenal mucosa became exhausted by a lavage of the duodenum with two liters of 0.2 per cent hydrochloric acid for a period of 3 to 4 hours. The question arises as to whether the intestinal mucosa remains in a normal condition when irrigated with acid for several hours. The data from only 4 of the 8 acid treated animals were published and the data indicate only the percentage increase in blood sugar concentration, so that one is unable to determine whether the fasting blood sugar concentration was within normal limits when the glucose was administered in the various experiments.

It has been reported (5) that hyperglycemia did not occur during absorption of glucose from the human stomach when the duodenum was being stimulated with dilute acid and other substances. However, hyperglycemia did occur in the absence of stimulation of the duodenum when an amount of glucose comparable to that absorbed from the stomach was placed directly into the duodenum. This observation may suggest that a duodenal hormone prevented hyperglycemia during duodenal stimulation. This report, as well as the more recent claim (15) that remarkably large and variable amounts of glucose were absorbed from the stomach of anesthetized dogs with a concomitant hyperglycemia in the absence of duodenal stimulation, remain to be confirmed.

The experiments designed to determine whether acid stimulation of the duodenum exerted any influence upon the ability of the animals to utilize liberal amounts of intravenous glucose yielded data which were strictly comparable to those which several investigators (12, 13, 14) have secured from extensive studies upon normal dogs. Therefore, these data indicate that the saline instilled in the duodenum in the control experiments induced no demonstrable alteration in carbohydrate metabolism. It is quite improbable that saline in the duodenum exerted any influence upon the blood sugar level in any of the other control experiments for our previously published data (1) indicate that saline was without effect upon the fasting blood sugar concentration.

The instillation of acid into the duodenum also failed to influence the

hyperglycemia induced by intravenous injections of adrenalin. The data obtained in these experiments confirm the results obtained by Shay *et al.* (5) who report that instillation of 0.51 per cent hydrochloric acid into the duodenum did not affect the hyperglycemia produced in 10 normal humans by injecting adrenalin chloride. The data from the experiments concerning adrenalin hyperglycemia and glucose utilization remove the possible criticism that the presence of glucose in contact with the intestinal mucosa obscured the hypoglycemic action of acid stimulation of the duodenum during alimentary hyperglycemia.

The experiments conducted upon nembutalized, depancreatized dogs yielded data which do not confirm the report made by LaBarre and Ledrut (3). Our experiments differed from theirs only in that nembutal was used instead of chloralose and the animals received 50 cc. of 0.3 per cent hydrochloric acid instead of 80 to 100 cc. of 0.5 to 0.8 per cent acid; a strength of acid which can scarcely be considered as simulating the normal acidity of the duodenal contents. The data which we have obtained from a larger series of animals fail to reveal such reductions in hyperglycemia.

The evidence secured from diabetic humans offers little, if any, support to the contention that duodenal instillation of dilute acid reduces an existing hyperglycemia. Shay *et al.* (5) quote Conti (4) as reporting that the hyperglycemia of diabetics was reduced by oral and duodenal administration of dilute acid. Boattini (6) controlled his experiments upon human diabetics by securing fasting blood sugar curves in the presence and absence of acid stimulation of the duodenum, but it remains doubtful whether his data warrant the conclusion that the acid stimulation was responsible for any decrease in blood sugar. However, the fact that the average blood sugar levels at the end of the three-hour periods were nearly identical in the test and control group suggests that spontaneous decreases occurred.

The four types of experiments conducted upon dogs have failed to yield data which indicate that acid stimulation of the duodenum influences carbohydrate metabolism. The experimental and clinical literature offers very little data which are at variance with our findings. Furthermore, our previous experimental studies (1) concerning the effect of acid stimulation upon the fasting blood sugar concentration gave only negative results. Therefore, we conclude that *acid* stimulation of the duodenum has no influence, either humoral or nervous, upon carbohydrate metabolism as revealed by changes in the blood sugar level in dogs. There remains the possibility, however, that some substance present in the duodenum other than hydrochloric acid may act as a stimulus to the duodenal mucosa and liberate the proposed hormone. The ingestion of fat (olive oil) induces no significant reduction in the fasting blood sugar concentration of dogs (16) or human subjects (17, 18). No evidence was found in the literature which suggested that the presence of any other substances in the duodenum

affected the blood sugar level to a significant degree. We believe, therefore, that no substantial evidence exists upon which to base the concept that a hormone which aids in the control of carbohydrate metabolism is liberated by the duodenal mucosa in response to stimulation.

CONCLUSIONS

1. One hundred and eight experiments upon 62 dogs have failed to yield evidence which indicates that acid stimulation of the duodenum enhances the utilization of intravenous glucose or reduces the hyperglycemia produced by absorption of glucose, injection of adrenalin, or removal of the pancreas.

2. The failure to demonstrate that acid stimulation of the duodenum influences either the normal or elevated blood sugar level seriously questions the physiological basis which has been used to support the concept that a hormone which aids in the control of carbohydrate metabolism is liberated by the duodenal mucosa in response to stimulation.

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THE ERROR OF ESTIMATE OF THE BLOOD CELL COUNT AS MADE WITH THE HEMOCYTOMETER

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Our primary object was to investigate the error of the blood cell count as it is made in good routine practice, and not as it might be made with special apparatus in exceptional situations. The method generally employed in this country of diluting and shaking the blood in a small pipet and then placing a sample by capillarity into a counting chamber was employed.[1] Previously we had found that counts made by eye were not precisely reliable, and we therefore used a mechanical method for counting, employing the same principles as devised by Amory and described by Sanford (21). The field of the chamber to be examined was projected on very sensitive paper and an enlarged photograph (x220) obtained. Each cell in the photograph was then perforated with a stylus connected electrically with a recorder which registered the cell as it was pierced.

Erythrocyte count. The basic source of variability of the count as estimated from the enumeration of the cells in a designated area of the counting chamber arises from the random settling of cells in different parts of the chamber. This error we call the "error of the field." The theoretical distribution of cells in the field was elaborated by "Student" (23), whose work is frequently cited in American and English literature. According to his results, the number of cells falling within an area of designated size in the hemocytometer chamber follows the "Poisson distribution,"[2] and, accordingly, the standard deviation (σ) is equal to the square root of the mean ($\sqrt{m}$). The probability distribution of cells in the field and the relation $\sigma = \sqrt{m}$ had been given as a theoretic result

[1] The pipets were of the Thoma-Zeiss type (1 cc., approximately, for erythrocytes, 0.5 cc., approximately, for leukocytes), and the chambers were of the Bürker variety with Neubauer ruling manufactured by Hauser, Philadelphia, and distributed by A. H. Thomas, New York. All apparatus used was within standards specified by the U. S. Bureau of Standards.

[2] "Student" evaluated the distribution independently and did not recognize it as the Poisson (25).

earlier by Abbe[3] and was found later apparently empirically by Reichel (20). Our own experiments and findings are as follows:

Blood was taken by finger puncture, diluted in a pipet 1:200 with Hayem's solution, shaken by hand for a minimum of three minutes, and the counting chamber filled by capillarity. The entire field of 1 sq. mm. was photographed and the cells in each of the 400 constituent squares were counted. Ten such experiments were performed. In table 1 is a summary of the results for the ten experiments. It is seen that the ratio $\sigma/\sqrt{m}$, which, according to the theoretic Poisson distribution, should equal unity, is consistently less than this and averages 0.92.[4] That the variability of the distribution is less than that calculated from theory is

TABLE 1

Relationship of standard deviation and square root of mean (Poisson = 1): erythrocyte counts

Each experiment included counts of the cells in each of the 400 squares of the square millimeter field.

EXPERIMENT	m	σ_f	$\frac{\sigma_f}{\sqrt{m}}$
1	6.04	2.33	0.95
2	6.49	2.33	0.91
3	6.03	2.45	1.00
4	6.75	2.39	0.92
5	5.31	2.18	0.95
6	6.77	2.28	0.88
7	5.09	2.09	0.93
8	6.31	2.20	0.88
9	5.75	2.09	0.87
10	5.49	2.15	0.92
1–10			0.92

explicable on the basis of a crowding effect of the cells in the chamber (24). Whether or not this is the correct explanation, we accepted the finding

[3] We do not agree with Dahlberg (10) that Abbe's derivation was false. Dahlberg apparently understood erroneously that Abbe derived the rule for relative variability $V = 1/\sqrt{n}$ by assuming the Gaussian curve with $S.D. = 1$. Actually Abbe correctly stated the probability of k cells falling in a square to be $e^{-n}\frac{n^k}{k!}$ where n represented the mean. He then showed that when n is large, the probability of a deviation from the mean follows closely the Gaussian curve of error with $\sigma = \sqrt{n}$, and that therefore the relative variability $= 1/\sqrt{n}$.

[4] Regarding a detailed comparison of the distribution in the chamber field with the Poisson distribution by the method of the chi-square test, and a critique of this method, see Berkson, Magath and Hurn (3) and Berkson (2).

itself as definitive and the measure of the field error σ_f as $0.92\sqrt{m}$. If the standard deviation of the field is expressed relatively in the coefficient of variation as a percentage of the mean, we have $V_f = 100 \times \frac{0.92}{\sqrt{m}}$. More generally, if more than one field is enumerated, the coefficient of variation $V_f = 100 \times \frac{0.92}{\sqrt{n_b}}$, where n_b is the total number of blood cells enumerated. Thus it is seen that the error accounted for by the variability of the field is not dependent upon the number of hemocytometer squares but on the total number of blood cells counted. For an anemic blood of 2,500,000 cells per cubic millimeter, twice the number of hemocytometer squares must be counted to achieve as small an error from the field source as for a normal blood containing 5,000,000 cells per cubic millimeter.

The field error is fundamental, and so long as the hemocytometer is used for estimating the blood cell count, it is unavoidable and the minimum error to which the count may be considered subject. If the dilution of the blood is effected with the same large quantitative pipet and mixing performed separately in special mixing flasks, and the same standardized chamber with the same cover slip is always used, and special precautions are taken to duplicate procedures exactly, this may be practically the only effective error.[5]

But under the more usual conditions of making a blood cell count by diluting and mixing the blood in any small pipet of the Thoma type, chosen at random from a satisfactory set, and utilizing any representative standardized chamber at hand, the field error is not the only one to which the blood cell count as finally made is subject. For convenience of investigation we shall consider as a unit each of two errors in addition to the error of the field already discussed: 1, σ_c, the effective error of the chamber as a whole; 2, σ_p, the effective error of the pipet as a whole. The chamber error represents the differences in the number of blood cells *between* chambers due to differences in accuracy of calibration and differences involved in the procedures of filling these chambers. The pipet error represents differences *between* pipets due both to differences in accuracy of calibration of the pipets and the differences involved in the manipulations of filling, diluting and mixing.

It is impracticable to measure the error of the chamber σ_c independently and directly, but one can achieve the objective indirectly as follows: In

[5] Such are the methods used, for instance, in the classic observations of Bürker (6), and hence the variability of his repeated observations represents practically only the field error. The same is true of the observations of other workers who used similar methods, for example, Lyon and Thoma (13), Reichel (20), and Plum (19), in all of whose work repeated observations conform closely to the rule $\sigma = \sqrt{m}$.

an experiment in which both the field error (σ_f) and the chamber error (σ_c) are involved,

$$\sigma_{fc}^2 = \sigma_f^2 + \sigma_c^2 \tag{1}$$

where σ_{fc} is the standard deviation of the total error involved. Accordingly, the following experiment was performed to determine σ_c. From a single pipetful of blood prepared as described before, ten chambers were filled and a count made of the cells in eighty squares (each square 1/400 sq. mm.) of each chamber. For each series of ten counts the mean (m) and the standard deviation (σ_{fc}) were determined. This was repeated for twenty-five subjects, a single pipet and ten chambers for each subject. The results are summarized in table 2. For the entire experiment the mean was 485 and σ_{fc}^2 [6] was 903. We have then, for the chamber error:

$$\sigma_c = \sqrt{\sigma_{fc}^2 - \sigma_f^2} = \sqrt{903 - 0.92^2 \times 485} = 22.19$$

TABLE 2

Experiments to determine combined field and chamber error, erythrocyte counts

Each experiment included a sample from a single pipet distributed on ten chambers.

EXPERIMENT	m	σ_{fc}^2	EXPERIMENT	m	σ_{fc}^2
1	508.8	872	15	455.5	1903
2	542.9	1165	16	497.8	897
3	536.0	359	17	496.0	568
4	507.8	514	18	407.8	264
5	454.9	365	19	479.6	871
6	467.1	902	20	489.1	464
7	554.5	667	21	450.8	712
8	548.2	1904	22	399.0	459
9	507.8	2581	23	486.7	244
10	442.1	1171	24	403.6	934
11	561.8	1024	25	499.2	1413
12	429.2	358			
13	457.2	1018	1–25	485	903
14	547.7	944			

We found also that for counts of different magnitudes σ_c was proportional to the mean. The coefficient of variation measuring the chamber error in percentage is, then, from this experiment:

$$V_c = 100 \times \frac{\sigma_c}{m} = 100 \times \frac{22.19}{485.2} = 4.6 \tag{2}$$

[6] For each experiment of ten counts σ_{fc}^2 was determined as $\frac{\Sigma\Delta^2}{9}$ where Δ represents deviation from the mean count of the ten. For the entire experiment σ_{fc}^2 was taken as the average value of the twenty-five experiments.

As in the case of the chamber, it is impracticable to measure the error of the pipet independently and directly but it can be obtained by an experiment which will involve the field error, the chamber error, *and* the pipet error.

$$\sigma^2_{fcp} = \sigma^2_f + \sigma^2_c + \sigma^2_p \tag{3}$$

To determine σ_p, ten pipets were quickly filled from a single puncture and after dilution and shaking as before, a sample from each pipet was placed on a different chamber, a count of the number of cells in the eighty squares made, and the mean (m) and standard deviation (σ_{fcp}) determined for the ten observations. This was repeated for forty specimens (that is, there were 400 pipetfuls and chamberfuls in all). In table 3 are summarized

TABLE 3

Experiments to determine combined field, chamber and pipet error, erythrocyte counts

Each experiment included a sample from a single blood specimen taken into ten different pipets and a specimen from each pipet placed in a different chamber.

EXPERIMENT	m	σ^2_{fcp}	EXPERIMENT	m	σ^2_{fcp}	EXPERIMENT	m	σ^2_{fcp}
1	575.4	1073	15	490.4	1529	29	494.6	1314
2	433.5	3601	16	432.8	505	30	372.2	546
3	490.9	814	17	391.8	789	31	416.2	1479
4	422.0	2345	18	412.0	1257	32	433.1	1478
5	530.2	3637	19	464.6	1230	33	445.8	709
6	455.8	1706	20	452.8	622	34	557.0	1217
7	513.5	2056	21	483.6	689	35	453.4	942
8	436.5	866	22	579.5	1206	36	480.7	1506
9	517.8	1763	23	596.2	1973	37	562.0	1983
10	470.5	362	24	573.6	1432	38	448.4	878
11	545.4	1355	25	195.5	408	39	500.4	872
12	442.0	957	26	124.7	390	40	503.5	1579
13	395.7	1600	27	519.8	2090			
14	409.5	814	28	504.4	1383	1–40	463	1324

the results. The average value of σ^2_{fcp} was 1324 and the mean 463. Hence we have

$$\sigma_p = \sqrt{\sigma^2_{fcp} - \sigma^2_f - \sigma^2_c} = \sqrt{1324 - 0.92^2 \times 463 - (0.046 \times 463)^2} = 21.88$$

For the coefficient of variation in percentage we have

$$V_p = 100 \times \frac{\sigma_p}{m} = 100 \times \frac{21.88}{463} = 4.7 \tag{4}$$

When a blood count is estimated utilizing not a single pipet specimen but, say, two pipetfuls of the blood, with a sample from each placed on a separate chamber and the total count estimated from the mean of these, the errors of the pipet (σ_p) and chamber (σ_c) are diminished, since the

error of a mean is inversely proportional to the square root of the number of samples. Taking this into account and inserting the ascertained values for V_c and V_p we have from (3) for the error of the total count measured as the coefficient of variation in percentage

$$V_t = \sqrt{\frac{(0.92 \times 100)^2}{n_b} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}} \tag{5}$$

where n_b is the total number of blood cells counted, n_c is the number of separate chamber specimens utilized and n_p is the number of separate pipet specimens employed.[7]

Thus, as an example, if a single pipet is filled and on a single chamber the cells within eighty squares counted are found to be 500, $n_b = 500$, $n_c = 1$, $n_p = 1$, the estimate is 5,000,000 cells per cubic millimeter, and the error of the estimate as coefficient of variation is 7.8 per cent. In routine statistical practice it is common to regard an estimate as significantly determined within $\pm 2\sigma$ or $\pm 2V$, for outside of these limits only about 5 per cent of random counts will occur; hence, we would say such an estimate is determined significantly as 5,000,000 $\pm$ 16 per cent or between 4,200,000 and 5,800,000. Similarly, for a count of 4,000,000 cells per cubic millimeter made in the same way, $V_t = 8.0$ per cent, for a count of 3,000,000 cells $V_t = 8.5$ per cent, for a count of 6,000,000 cells $V_t = 7.6$ per cent, and so forth. If a count is made by filling a single pipet, charging two chambers and counting forty squares in each and a count of 500 cells is found, the estimated count is 5,000,000 per cubic millimeter, $n_b = 500$, $n_c = 2$, $n_p = 1$, and V_t is now 7.0 per cent instead of 7.8 per cent. If two pipets had been used and each charged on a separate chamber, in each of which eighty squares were counted, for a count of 5,000,000 per cubic millimeter, $n_b = 1000$, $n_c = 2$, $n_p = 2$, and $V_t = 5.5$ per cent.

It is notable from (5) that only a small fraction of the total error can be diminished by increasing the number of cells counted (n_b). There is, therefore, little point to counting the cells in a field greater than eighty squares in a single chamber. If greater accuracy is desired, the procedure should be to increase the number of pipet and chamber samples.

From what has been so far developed, there are important implications that bear on statements frequently made regarding the accuracy of repeated counts and on criteria which have been laid down for standardization of accuracy in counting. What we have dealt with relates to the error of an estimate of the count made under different defined conditions. From the error of a single estimate, we can also calculate by established statistical theorems the probability of expected *differences* between successive estimates. For instance, if ten successive counts are made from a

[7] This is for conditions in which the enumeration itself is effected without error, and hence does not include the error of the eye count (see Magath, Berkson and Hurn (15)).

sample of blood that contains 5,000,000 erythrocytes per cubic millimeter, the difference between the largest and the smallest of these will be 500,000 or more 99 per cent of the time, 1,000,000 or more 74 per cent, and 1,500,000 or more 17 per cent of the time. Published studies involving erythrocyte counts, as well as standard texts, disallow discrepancies between successive counts so small that they would in most instances necessarily be exceeded as a matter of chance if counts were accurately made and faithfully recorded. In table 4 are shown some representative criteria for maximum discrepancies to be allowed under a number of various conditions. In the adjoining column are comments giving the probabilities that such discrepancies will occur normally. What we find is that the basic maximum discrepancies allowed in standard works would normally be exceeded in anywhere from 66 to 85 per cent of the time. To us it appears that such rules for necessary agreement of repeated counts are meaninglessly stringent and serve only to encourage erroneous counting by requiring an attempt to live up to impossible standards.

Leukocyte count. As in the case of the erythrocytes, the basic error of the leukocyte count has its source in "the error of the field," which arises from the random distribution of cells in the hemocytometer chamber. For leukocytes, however, when dilution is made in the usual ratio of 1:20, the density of cells in the field is in the order of a hundredth of that which obtains in the chamber when erythrocytes are enumerated. It did not appear to us justifiable therefore to apply the rule which we had found for the field error of the erythrocytes ($\sigma_f = 0.92\sqrt{m}$) to the leukocytes without independent verification.

Accordingly a series of experiments was undertaken to ascertain directly the field error for leukocytes. Blood drawn from a vein of the forearm was used. Dilution in the ratio 1:20 was effected in a pipet with acetic acid (1 per cent) and the pipet was shaken by hand for a minimum of three minutes. The contents of the pipet were distributed into as many chambers as the volume of the pipet permitted, which was usually five, sometimes only four, two drops being expelled from the pipet between the filling of the chambers. The cells in each of the 9 sq. mm. of the entire chamber were enumerated[8] and from these nine counts the mean and

[8] A preliminary investigation was made to compare directly, for the leukocyte counts, enumeration as made by eye and that obtained by the photographic-mechanical method used in all the reported experiments dealing with erythrocytes. In contrast with what was found in a similar direct comparison for the erythrocytes, a practically perfect agreement was found between the eye and photographic counts of the leukocytes. This is not surprising, considering that the leukocytes are distributed with a density only a hundredth of that of the erythrocytes. Accordingly, we did not insist, in the experiments dealing with the leukocytes, that the counts be made by the photographic method. The enumerations for some of the experiments were made by the photographic method but most of them by eye count. One of the investigators (Hurn) performed almost all the enumerations.

TABLE 4

Representative criteria for maximum discrepancies to be allowed in erythrocyte counts

CRITERION	AUTHOR	COMMENT
Allows c. of v. of from 0.2 to 3 per cent for counts of eighty squares, successive drops from pipet	C. Smith (22)	The c. of v. under these conditions should be about 6 per cent
Two successive counts should agree within 300,000 per cubic millimeter	Cummer (9)	A greater disagreement than this can be expected in 66 per cent of normal counts
Count the cells in a row of twenty small squares. . .then count twenty more squares until five rows of twenty squares have been counted. The highest and lowest total should not vary by more than twenty	E. E. Osgood (18)	Such variation may be expected normally in 67 per cent of the cases
Red blood cell counts were made on at least two different dilutions from each sample of blood, and the average of two counts differing by less than 200,000 was taken as the value	E. R. Mugrage and M. I. Andresen (16)	Greater differences are to be expected 77 per cent of the times
Count the blood of one person daily at the same hour until the difference between the counts of two successive days is not over 200,000 and the difference between the highest and lowest counts of the eight units (each unit containing twenty-five small squares) for each day is not over twenty-five cells	C. P. Emerson (11)	The difference between successive days can be expected in 77 per cent and that between units in 84 per cent of normal counts*
The normal routine in counting erythrocytes was for each individual to count each sample until duplicate determinations agreeing within 100,000 cells had been obtained	C. F. Nelson and R. Stoker (17)	A difference larger than the one allowed can be expected in 85 per cent of normal counts
Two dilutions of 1:200 were counted in each case. Only results agreeing within 100,000 were accepted	M. M. Wintrobe and M. W. Miller (26)	Under this rule 85 per cent of normal counts would not be acceptable

* Emerson says: "Some students attain this accuracy quickly. Some students, however, repeat this daily, counting for from 20 to 30 or even more days before their work is satisfactory to themselves or to us. By this time they certainly have learned wherein lies the error of their technic. It is of interest that the most careful ones sometimes make the great errors, since they take too much time where speed is essential." To the present authors this appears as evidence that the students are thus taught to develop an unconscious habit of making counts agree.

standard deviation were determined.[9] Fifteen such experiments were made and the results are summarized in table 5A. It is seen that the ratio of the standard deviation to the mean ($\sigma_f/\sqrt{m}$) is not consistently less than unity as we found for the erythrocytes, and the average is very nearly unity, as it should be for the theoretic Poisson distribution. This difference of the field error of the leukocytes from that of the erythrocytes may be ascribed to the comparative sparsity of the leukocytes in the chamber. We accepted these results as definitive and so took the error

TABLE 5

Relationship of standard deviation and square root of mean (Poisson = 1): leukocyte counts

Each experiment included four or five repetitions of counts from a single pool of blood, made in each of the nine square millimeters of the hemocytometer chamber.

EXPERIMENT	A. PIPETS SHAKEN BY HAND				B. PIPETS SHAKEN BY ROTOR			
	Number	m	σ_f	$\sigma_f/\sqrt{m}$	Number	m	σ_f	$\sigma_f/\sqrt{m}$
1	5	28.4	5.79	1.09	4	27.9	4.63	0.88
2	4	57.3	6.83	0.90	5	44.5	7.44	1.12
3	5	40.3	6.63	1.04	5	29.1	4.60	0.85
4	5	30.3	4.82	0.88	5	33.5	6.22	1.07
5	4	36.2	6.07	1.01	4	26.2	4.23	0.83
6	4	40.0	6.10	0.96	5	46.8	6.42	0.94
7	5	30.4	6.30	1.14	5	46.1	6.77	1.00
8	5	27.4	5.43	1.04	4	26.3	4.68	0.91
9	5	56.5	8.25	1.10	5	30.9	6.09	1.10
10	5	46.8	7.10	1.04	5	44.6	6.43	0.96
11	5	44.4	6.02	0.90	5	39.0	6.68	1.07
12	4	100.8	11.34	1.13	5	40.4	7.36	1.16
13	4	29.2	5.24	0.97	4	47.7	8.75	1.27
14	5	25.2	5.07	1.01	5	39.2	5.99	0.96
15	5	43.1	7.96	1.21	5	37.8	5.04	0.82
1–15				1.03				1.00

of the field as $\sigma_f = \sqrt{m}$ for the leukocyte counts; accordingly the coefficient of variation in percentage is

$$V_f = \sigma_f/m \times 100 = 100/\sqrt{m} \qquad (6)$$

[9] For each chamber count consisting of the nine observations, the standard deviation was determined as $\sigma_f = \sqrt{\frac{\Sigma\Delta^2}{8}}$ where Δ represents deviation from the mean. For each experiment as a whole, consisting of five such determinations, the standard deviation was determined as $\sqrt{\frac{\Sigma\sigma_f^2}{5}}$.

With the error of the field (σ_f) evaluated, we assumed provisionally that the error of the chamber found for the erythrocytes (4.6 per cent) and that for the pipet (4.7 per cent) could be used for the leukocytes, since actually the same chambers and the same type of diluting pipet are employed. Accordingly, for leukocytes the error of the count measured in percentage is given by (7):

$$V_t = \sqrt{\frac{100^2}{n_b} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}} \qquad (7)$$

TABLE 6

Comparison of total error (V_t) observed and calculated for leukocyte counts

Cells in 4 sq. mm. counted. Ten pipets and ten chambers from a single blood specimen in each experiment.

EXPERIMENT	A. PIPETS SHAKEN BY HAND				B. PIPETS SHAKEN BY ROTOR			
	m	σ_t	V_t—per cent		m	σ_t	V_t—per cent	
			Observed	From formula (7)			Observed	From formula (7)
1	152.4	18.5	12.1	10.4	140.8	14.9	10.6	10.7
2	158.0	14.9	9.4	10.3	197.2	13.0	6.6	9.7
3	88.3	9.9	11.2	12.5	180.6	18.8	10.4	9.9
4	125.7	12.7	10.1	11.1	136.4	13.3	9.8	10.8
5	204.9	15.9	7.8	9.6	71.1	10.7	15.0	13.6
6	113.7	11.8	10.4	11.5	108.2	9.9	9.1	11.6
7	182.5	24.2	13.3	9.9	158.2	12.9	8.2	10.3
8	123.9	17.7	14.3	11.1	129.5	16.5	12.7	11.0
9	91.9	11.9	12.9	12.3	112.8	13.6	12.1	11.5
10	165.3	14.1	8.5	10.2	136.8	13.3	9.7	10.8
11					142.0	19.4	13.7	10.7
12					106.4	8.8	8.3	11.7
13					135.1	11.0	8.1	11.2
14					157.1	20.7	13.2	10.3
15					127.4	18.1	14.2	11.0
Total			11.0	10.9			10.8	11.0

where n_b is the total number of blood cells counted, n_c the number of chambers used, and n_p the number of pipets.

To test the validity of formula (7), ten pipet samples were taken from a single pool of blood preliminarily placed in a wax cup, and from each pipet sample, in a separate chamber, an enumeration was made of the cells within the area of four large squares (each square 1 sq. mm.). Ten such experiments were performed and in each the coefficient of variation was compared with that given by (7). The results are summarized in table 6A. It is seen that there is a reasonably close agreement: there is a fluctuation in the sign of the difference between observed and expected,

which we may regard as sampling variation, and the average shows a coefficient of variation observed as 11.0 per cent, as against a predicted percentage of 10.9.

As an example, from (7), if a single specimen of blood is taken and diluted in 1:20 ratio, and a count made over 4 sq. mm. of the hemocytometer field yields 140, the estimate for the mean leukocytes per cubic millimeter is 7,000 and $V_t = 10.7$ per cent ($n_b = 140$, $n_p = n_c = 1$). We may say such a count is subject to an experimental error of $\pm$ 21.4 per cent (twice the coefficient of variation). If the single pipet specimen is distributed in two chambers and the count for the 8 sq. mm. in the two fields examined yields 280 leukocytes, the mean count is estimated as 7,000 cells per cubic millimeter, $V_t = 8.3$ per cent ($n_b = 280$, $n_c = 2$, $n_p = 1$), and the estimate is significantly determined within limits of $\pm$ 16.6 per cent.

The magnitude of the errors which we are reporting here is smaller than that of some which have appeared in the literature,[10] but setting it against what we consider the most careful comparable studies it is generally larger. Particularly we were impressed by the differences from those reported by Bryan and Garrey (4), and Bryan, Chastain and Garrey (5). Bryan and Garrey, working with leukocytes, found that shaking the pipet by hand to mix the cells, unless performed in a certain specified manner, was inadequate and that a large variability in the counts can result if improper shaking is used. However, when they used a machine designed by them to shake the pipet, a much smaller dispersion of the counts resulted regularly, which they attributed to the more adequate shaking effected by their machine. For the one experiment in which their observations are given in sufficient detail (p. 1060, table 2) (4) to permit direct calculation of the "field error," we find the ratio of $\sigma/\sqrt{m}$ to be 0.75 instead of unity, which is the theoretic value, and which we found to obtain in the case of leukocytes. This is of course only one series and might be a random variation; however, it consists of nineteen separate chamber specimens. Moreover, in another large series of tabled experiments by Bryan, Chastain and Garrey (p. 421, table 1) (5), employing the same technic, though details permitting a direct calculation of $\sigma/\sqrt{m}$ are not available, an indirect calculation from the constants given indicates an average value for $\sigma/\sqrt{m}$ much below unity.

It did not appear to us that shaking of the pipet by machine should so affect the field error.[11] Even assuming that inadequate shaking of the pipet results in an ununiform dispersion of cells *in the pipet*, it would seem that the small fraction that is admitted to the counting chamber, while

[10] For instance, we calculated from table II of Yates and Batt (27) the total error V_t as 15.3 per cent; we should have expected 8.5 per cent (formula 7).

[11] However, Macfarlane (14) too, by employing a shaking machine achieved a field dispersion for which we calculated $\sigma/\sqrt{m} = 0.46$.

unrepresentative and hence unreliable for an estimate of the total count, would be spread more or less at random *in the chamber* and hence give a field error $\sigma_f = \sqrt{m}$.

It appeared imperative to compare by direct experiment the results we obtained with what would be given if we utilized shaking of the pipet by machine. A shaking machine of the Bryan-Garrey design was utilized,[12] and a series of experiments made, to evaluate the error of the field, following exactly the method used when the pipets were shaken by hand. The pipetful of blood after dilution was shaken in the machine for a minimum of ten minutes and spread upon as many chambers as the contents permitted. In each chamber the cells in each of the 9 sq. mm. were enumerated,[13] and σ_f and m determined. From the mean values of m and σ_f^2 for the four or five samples of the pipet, the ratio $\sigma/\sqrt{m}$ was calculated. The results for fifteen such experiments are summarized in table 5B. It is seen that the values for $\sigma/\sqrt{m}$ fluctuated around unity, and, as it happens, the average was unity precisely. This experience verifies the results for σ_f which we obtained with hand shaking.

We were able furthermore in a number of instances to find details of the results of other workers which permitted a calculation of $\sigma_f/\sqrt{m}$. Thus some experiments of Bürker's (7) with erythrocytes published in detail, in which each count of 320 small squares is given, yield upon calculation a value for $\sigma_f/\sqrt{m} = 0.93$. A similar series for erythrocytes of Craandyk (8), giving the individual counts in each of 200 squares, yields $\sigma_f/\sqrt{m} = 0.94$. A summary table giving the distribution of leukocyte counts in the hemocytometer field by Yates and Batt (p. 166, table IV) (27) yields $\sigma_f/\sqrt{m} = 0.99$.[14]

For the total error of the leukocyte count also our results give a larger value than that reported in the results of Bryan, Chastain and Garrey. Whereas our results indicate for a mean count of 7,000 per cubic millimeter a coefficient of variation of about 11 per cent, their results indicate an error in the order of 4 per cent for the "compound variable" which apparently corresponds to our "total error." Moreover, they concluded that the standard deviation is independent of the mean count whereas, according to our formula (7), the standard deviation is correlated with the

[12] A. S. Aloe Co., St. Louis, Mo., manufacturer.

[13] The relevant experiments of Bryan and Garrey, and of Bryan, Chastain and Garrey were performed in the same way except that more chamber specimens were available from the large pipet they used.

[14] Faergeman (12) calculated σ_f from a series of 234 duplicate counts in two large fields of the same chamber. For the mean he obtained 468, and for the standard deviation 22.81. Thus $\sigma/\sqrt{m} = 1.05$, that is, *larger* than unity. However, the result is based on only 234 effective observations (as contrasted, for instance, with the ten experiments each of 400 observations in our series) and the value obtained for $\sigma/\sqrt{m}$ is not significantly different statistically from our average of 0.92.

mean. We therefore performed a series of fifteen experiments each consisting of ten counts, employing the Bryan-Garrey rotor for shaking, to obtain the total error in the same way that it was calculated from our experiments when hand shaking was employed. We found (table 6B) an average value for V_t of 10.8 per cent, to be compared with a predicted value of 11.0 per cent from formula (7). The results of this experiment corroborate the results for the total error obtained when hand shaking was employed.

What the explanation of these discrepancies in results is we are not sure. In one instance in which the results with erythrocyte counts of another author gave for the field error a ratio of $\sigma_f/\sqrt{m}$ very much less than unity, we had the opportunity to examine photographs of the cells in the field which he had enumerated. When we enumerated these cells in each of the small constituent squares of the rows with a mechanical counter, we obtained for $\sigma_f/\sqrt{m}$ values which verified the theoretic value of unity very closely. Apparently in this instance there had been an unconscious tendency to diminish the differences between successive counts. For the leukocytes, where the cells are so widely dispersed and comparatively few are counted, it seems improbable that such biased counting should occur, but the possibility cannot be excluded.

As far as our "total error" is concerned, it should be understood that this represents the error to which a count is subject when it is made with a pipet and chamber taken at random. Hence, an experiment which is designed to measure this error must employ a number of different pipets and chambers in the same experiment (with us the number was ten in each such experiment). It is not always clear whether experiments designed to estimate this total error do effectively include the variabilities represented in *different* pipets and *different* chambers.

SUMMARY

The error of an individual determination of the blood cell count when the enumeration itself is effected without error, measured as coefficient of variation in percentage, can be estimated from (5) for erythrocytes and from (7) for leukocytes:

$$V_t = \sqrt{\frac{(0.92 \times 100)^2}{n_b} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}} \qquad (5)$$

$$V_t = \sqrt{\frac{100^2}{n_b} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}} \qquad (7)$$

$\sqrt{\frac{(0.92 \times 100)^2}{n_b}}$ is the "field error" for erythrocytes measured as coefficient of variation in percentage; $\sqrt{\frac{100^2}{n_b}}$ is the "field error" for leukocytes mea-

sured as coefficient of variation in percentage; n_b is the total number of blood cells counted; 4.6 is the "error of the chamber" measured as coefficient of variation in percentage, and n_c is the number of chamber specimens examined; 4.7 is the "error of the pipet" measured as coefficient of variation in percentage and n_p is the number of separate pipet specimens employed.

Accordingly for a count of 5,000,000 erythrocytes per cubic millimeter obtained from a single specimen sample counted in eighty small squares, $V_t = 7.8$ per cent ($n_b = 500$, $n_c = 1$, $n_p = 1$). We may say, following a usual practice of taking two times the standard error as significant limits, that such a count is determined significantly within $\pm$ 16 per cent. Similarly for a leukocyte count of 7,000 per cubic millimeter obtained by counting 4 sq. mm., $V_t = 10.7$ per cent ($n_b = 140$, $n_c = 1$, $n_p = 1$) and the count is determined significantly within $\pm$ 21 per cent. For counts obtained by using more than one chamber or pipet sample, V_t and the significant limits can be determined by inserting the appropriate numbers for n_b, n_c and n_p in equations (5) and (7).

Representative standards laid down in texts for the necessary agreement between successive counts taken in series are meaninglessly stringent. Differences considered as too large to be allowed will occur very frequently (66 to 85 per cent of the time) if counting is made precisely and recorded faithfully.

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RELEASE OF AUTONOMIC HUMORAL SUBSTANCES IN HYPOGLYCEMIC CATS AND MONKEYS

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Insulin hypoglycemia is accompanied by widespread changes in body functions. Hyperactivation of the autonomic nervous system, with the release of hormones into the blood stream, is one of the effects already demonstrated (Cannon, McIver and Bliss, 1924; Partington, 1934). The humoral response of the sympathetic division of the autonomic nervous system to hypoglycemia has been established by many investigations. On the other hand, little or no attention has been paid to the influence of hypoglycemia upon the parasympathetic division of the same system.

In this work an effort has been made to study the humoral responses of both divisions of the autonomic nervous system to insulin hypoglycemia. Experiments were made upon the denervated structures of the cat and monkey in accordance with the principle that such structures are hypersensitive to hormones of the autonomic nervous system and may, therefore, be used as indicators for the presence of adrenaline and acetylcholine (Bender, 1938).

MATERIALS AND METHODS. Forty cats and eleven monkeys (macaca mulatta) were used. The presence of sympathomimetic hormones in the cat was detected by dilatation of the pupil, retraction of the nictitating membrane and constriction of the ear vessels, all sensitized to adrenaline by cutting the post ganglionic fibers of the cervical sympathetic. The pupillary dilator fibers of the monkey were rendered sensitive to adrenaline in a similar fashion. The denervated facial muscles of the cat and monkey were employed to indicate the parasympathomimetic hormone (Bender and Kennard, 1938).

In fifteen cats and five monkeys the superior cervical ganglion and the facial nerve were removed on the right and left sides, respectively.

An additional indicator of humoral autonomic substances employed was the completely denervated iris. This pupil is dilated by adrenaline and constricted by acetylcholine. In twenty cats and four monkeys the optic and all ciliary nerves were sectioned in one eye. The operation was a modification of that used by Anderson (1905), Shen and Cannon (1936).

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All operations were performed with aseptic technic and under nembutal anesthesia. Hypoglycemia was induced by the parenteral injection of insulin from two to six units, per kilogram of body weight. In ten instances the observed effects were controlled by blood sugar determinations. The fasting blood sugar levels were 100 to 90 mgm. per 100 cc. of blood. The lowest levels reached in hypoglycemia were 30.0 mgm. per 100 cc. of blood. In almost all experiments the hypoglycemia was severe enough to produce convulsions. The presence of convulsions was used to indicate the depth of hypoglycemia. Glucose by mouth or vein invariably abolished the above symptoms.

RESULTS. I. *Cats:* A. *Sympathetic humoral responses.* 1. Sectioning of the post ganglionic sympathetic nerve supply to the iris of the cat rendered the dilator fibers hypersensitive to adrenaline circulating in physiologic amounts. As little as 0.08 gamma of adrenaline per kilogram of

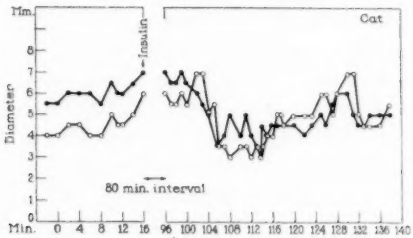

Fig. 1. Effect of insulin hypoglycemia on the denervated pupil. Cat 3.2 kgm. Insulin 13 units intramuscularly. Right superior cervical ganglion excised 12 days previously. Ordinate: Horizontal diameter of pupil; solid dots represent normal and circles denervated pupils respectively. Abscissa time in minutes.

body weight injected into the saphenous vein produced dilatation of this sensitized pupil. Such dilatation has been termed the paradoxical pupillary reaction (Langendorf, 1900). Enlargement of the s.d.[2] pupil in the course of insulin hypoglycemia may thus be accepted as presumptive evidence of the release of epinephrine.

Before hypoglycemia was induced or in the resting cat, the diameter of the s.d. pupil indicated by circles in figure 1, was always smaller than the normal, indicated by heavy dots. This difference in size between the two pupils was constantly maintained during variations normally caused by reflex light and other constrictor or dilator mechanisms.

In insulin hypoglycemia, the s.d. pupil usually began to dilate sixty minutes after the intramuscular injection of insulin. First it grew larger

[2] For brevity the post ganglionic sympathetically denervated pupil will be designated as s.d. pupil, the completely denervated pupil as c.d. pupil and the nictitating membrane as n.m.

and then smaller than the normal pupil. These fluctuations in the diameter of the s.d. and the normal pupils are illustrated in figure 1. A similarly fluctuating response to hypoglycemia has been noted by Cannon et al. (1926) in experiments using the completely denervated heart as an indicator. The iris denervated by preganglionic section was insensitive to physiologic doses of adrenaline and failed to dilate in the hypoglycemic cat.

2. The denervated nictitating membrane (n.m.) retracts with insulin hypoglycemia (Partington, 1936). The normal n.m. does not retract as early or as much as the denervated. In our experiments the retraction usually appeared forty-five minutes after the injection of insulin and the earliest retraction of the n.m. was found to be within thirty minutes.

3. Cooling of the denervated ear was repeatedly felt in the course of insulin hypoglycemia. The effect first appeared at the end of thirty minutes. This occurred before any fall in body temperature and while the normal ear remained warm. In several cats there was a simultaneous constriction of the denervated ear blood vessels. This was manifest by a conspicuous blanching in the denervated ear.

B. *Parasympathetic humoral responses.* Denervated facial muscles of monkeys are sensitive to circulating acetylcholine (Bender, 1938). However, in order to visualize clearly the contractions in denervated facial muscles of the cat, large amounts of acetylcholine were necessary so that at best it was not a sensitive parasympathetic indicator in this species.

In our experiments, three cats displayed some contraction in the denervated face. This was most conspicuous in the m. orbicularis oculi which practically closed the palpebral fissure and occurred when the denervated facial muscles were most sensitive to acetylcholine, usually twenty-eight days after denervation. The contractions in facial and eyelid muscles prevailed at the height of and after the appearance of sympathetic effects.

II. *Monkeys:* A. *Sympathetic humoral responses.* As in the cat, the sympathetically denervated pupil of the monkey was sensitive to adrenaline but the sensitivity was low. In contrast with the findings in the cat, the sensitized pupil in the macaque did not dilate in the course of insulin hypoglycemia. It dilated in hypoglycemia only if cocaine was instilled into the conjunctival sac or when administered parenterally in large doses, i.e., 8 mgm. per kilogram of body weight. In the resting state or in the absence of hypoglycemia the s.d. pupil either did not enlarge at all with cocaine or at most, only incompletely. This is illustrated in figure 2. Three drops, one drop every five minutes, of two per cent cocaine hydrochloride were instilled in each of the conjunctival sacs. The normal pupil, indicated by heavy dots, dilated while the s.d. pupil, indicated by circles, remained practically unaltered. In insulin hypoglycemia, however, the instillation of only one drop of the same solution of cocaine produced bilateral mydriasis and more so of the pupil sensitized by post ganglionic sympathetic denervation.

B. Parasympathetic humoral responses. The denervated facial muscles of the macaque are seemingly more sensitive to acetylcholine than those of the cat. However, in three monkeys only slight contractions in the denervated facial muscles occurred in the course of hypoglycemia. This is, at most, only suggestive evidence that a cholinergic substance is discharged in response to hypoglycemia in this species.

III. *The completely denervated pupil in the cat and monkey.* Because of the possibility that the dilatation of the s.d. pupil in hypoglycemia is the result of inhibition of the oculomotor pupillary tone (Ury and Gellhorn,

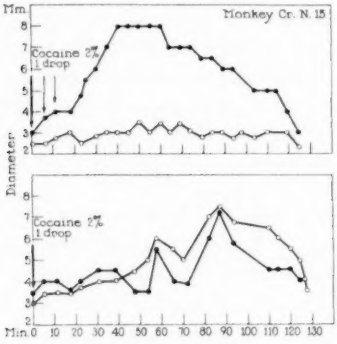

Fig. 2

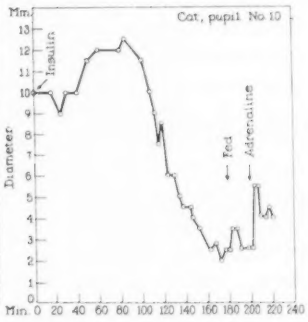

Fig. 3

Fig. 2. Monkey 3.0 kgm. Right superior cervical ganglion removed 28 days previously. Ordinate: Horizontal diameter of pupil; solid dots represent normal and circles denervated pupils respectively. Abscissa time in minutes.

A. Effect of one drop of cocaine 2 per cent applied thrice locally.

B. Effect of one drop of cocaine 2 per cent applied once locally during hypoglycemia, one hour after the intramuscular injection of 14 units of insulin.

Fig. 3. Cat 3.8 kgm. Insulin units 16 intramuscularly. Right ciliary and optic nerves resected eight days previously. Ordinate and abscissa same as in above graphs. Circles represent horizontal diameter of the right pupil.

1939) or due to impulses carried by the ophthalmic division of the trigeminus (Bender and Kennard 1938), all the nerves entering the eyeball were destroyed, thus producing a totally denervated pupil. In the cat, such a pupil was found to be sensitive both to adrenergic and cholinergic substances. The smallest dose of adrenaline which evoked dilatation was 0.04 gamma per kilogram of body weight, injected into the saphenous vein, as compared with 0.08 gamma for the s.d. pupil. With acetylcholine the responses of this pupil in the cat were variable, for not only was there a constriction but on occasions delayed dilatation. Since this iris was devoid of nervous control, any change in the diameter of the pupil must have been due to a circulating substance, either dilator (adrenergic) or constrictor (cholinergic) in its action.

In the cat hypoglycemia usually caused the totally denervated pupil to undergo dilatation. The earliest at which the expansion became visible was twenty-five minutes after the injection of insulin. The mydriasis persisted for many minutes. In four cats, however, the phase of dilatation was followed by marked and prolonged miosis. This is well demonstrated in figure 3. At first the diameter of the c.d. pupil increased and then decreased rapidly. With the pupillary constriction there was hyperemia and edema of the ipsilateral conjunctiva. This engorgement was apparently due to a dilatation of the conjunctival blood vessels.

The parasympathetic phenomenon, bradycardia, which is well known to occur in hypoglycemia, was marked in our experimental animals. At times the heart rate dropped from a basal level of 180 beats down to 90 or 80 per minute in hypoglycemia. Although bradycardia and mydriasis of the denervated pupil were constant findings in hypoglycemic cats, constriction of the c.d. pupil was not. In a majority of the cats there was mydriasis of the c.d. pupil, and only in five was there miosis and congestion of the conjunctiva in the completely denervated eyeball.

After the removal of both adrenal glands, the paradoxical pupillary reaction in hypoglycemia was abolished. Instead a slight or marked constriction of the c.d. pupil ensued. This is illustrated in figure 4. With the adrenals intact, there is dilatation and with the adrenals removed, there is rapid and marked constriction of the c.d. pupil in hypoglycemia. The phenomenon of constriction of the c.d. pupil in the hypoglycemic cat cannot be elicited until the denervated circular fibers of the iris are hypersensitive to acetylcholine.

In four monkeys with c.d. pupils, hypoglycemia produced no enlargement of the pupil. In one of these, there was constriction of the c.d. pupil, see figure 5.

Discussion. That a discharge of an adrenergic substance occurs in the cat during insulin hypoglycemia has already been demonstrated, especially by Cannon, McIver and Bliss, 1924, who employed the denervated heart and the denervated nictitating membrane as indicators. This conclusion is confirmed by the present observations on the sympathetically denervated pupil, nictitating membrane and ear vessels, and upon the completely denervated pupil. Removal of both adrenals, or excision of one and denervation of the other, have been shown to diminish or abolish this effect.

The demonstration of a sympathomimetic response to hypoglycemia in the monkey (macaca mulatta) is much more difficult because the s.d. pupil in the monkey is not very sensitive to adrenaline, and during hypoglycemia it dilates only after the administration of cocaine. The amount of adrenaline discharged in the hypoglycemic macaque may be as great as in the cat, but the denervated radial fibers of the macaque's iris are not as chemically excitable and therefore do not clearly indicate the

presence of epinephrine. All that the available data at hand seem to indicate is that a substance in the hypoglycemic monkey predisposes the s.d. pupil of the monkey to dilate with cocaine. It is only assumed that this secretion is adrenaline or a related substance, because the effect of adrenaline is potentiated by cocaine (Rosenblueth and Rioch, 1932).

The s.d. pupil of man is likewise less responsive to epinephrine than that of the cat. An opportunity to observe the effects of adrenaline on the iris deprived of its sympathetic supply in man occurred in a patient who had a malignant neoplastic destruction of the cervical sympathetic chain and carotid plexus. This pupil was found to dilate but slightly in response

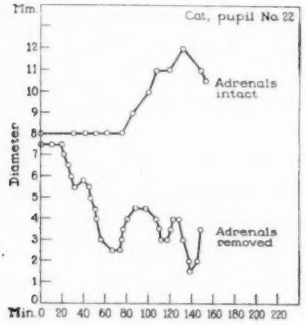

Fig. 4

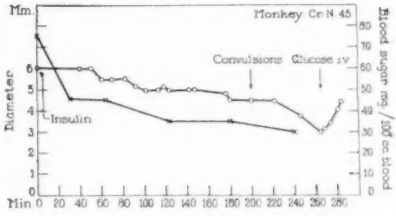
Mm.
Monkey Cn N. 45
Convulsions
Glucose i.v.
Insulin
Diameter
Blood sugar mg. / 100 cc. blood
Min. 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280

Fig. 5

Fig. 4. Cat 4.1 kgm. Right ciliary nerves and ganglion resected on June 29, 1939. A. July 5, 1939, insulin, 16 units, was given intramuscularly. Fifteen minutes later the c.d. pupil indicated by circles, dilated.

B. July 6, 1939, bilateral adrenalectomy. July 7, 1939. Insulin 16 units intramuscularly and at end of twenty minutes, the pupil began to constrict. Ordinate and abscissa same as in previous figures.

Fig. 5. Monkey 2.8 kgm. Insulin units 12 intramuscularly. Right ciliary and optic nerves resected 35 days previously. Blood sugar represented by crosses. Ordinate: Horizontal diameter of pupil indicated by circles. Note the steady decrease in pupillary diameter as the blood sugar falls and the increase in diameter on administration of glucose by vein. Abscissa: time in minutes.

to 200 gammas of intravenous adrenaline (3.0 gamma per kgm. of body weight). In the same patient marked insulin hypoglycemia induced no pupillary responses whatsoever. That hypoglycemia evokes an adrenergic response in man is indicated by observations made on denervated peripheral vessels by Freeman, Smithwick and White (1934) and on the perfusion of human hypoglycemic blood through severed rabbits' ears and other sensitized test organs by Brandt and Katz (1933).

It is apparent then that the cat, unlike other animals, possesses structures which when denervated serve as excellent indicators of the presence of sympathomimetic substances. However, because of these conspicuous

adrenergic responses, cholinergic effects may be overlooked, particularly since acetylcholine is so rapidly destroyed by cholinesterase. Nonetheless, the observations here presented testify that some parasympathetic hormonal effects do occur in the course of hypoglycemia. The contraction response of the denervated face of the cat, and in the monkey, which presumably are parasympathetic responses are not marked and are inconstant. More convincing evidence that acetylcholine, or some similar agent, is discharged in the body in response to insulin hypoglycemia, is found in the observations made upon the completely denervated pupil. The secondary miosis of this pupil, observed in several cats and one monkey, must be due to a cholinergic substance discharged locally or circulating in the blood stream. Whether this chemical is acetylcholine itself remains somewhat problematical in the light of the prolonged effects noted. As already mentioned, acetylcholine is so rapidly destroyed that its action is necessarily brief. Nevertheless, we found that the constriction of the c.d. pupil reproduced by intravenous acetylcholine was prolonged. Clarke and Raventos (1938) showed that acetylcholine may continue to act on the cell by adsorption long after it disappears from the blood stream.

It is interesting that the cholinergic effects appeared late in hypoglycemia, at a distinct interval after the adrenergic phenomena (see fig. 3). It is possible that in accordance with the homeostasis theory (Cannon, 1938), the continual liberation of adrenaline produces a reflex discharge of acetylcholine thus tending to maintain a balance between the sympathetic and parasympathetic systems in the humoral as well as in the neural spheres. The marked vagal slowing of the heart which is so constant a feature of late hypoglycemia suggests that the liberation of acetylcholine into the general circulation occurs at the vagus nerve-endings and that the humoral cholinergic phenomena of hypoglycemia occur in response to the hyperadrenalemia. Study of the rôle of the vagi in hypoglycemia might offer some evidence in this direction.

The absence of paradoxical pupillary reaction and the persistence of cholinergic phenomena after the removal of both suprarenals is against the theory that adrenaline stimulates the production of a cholinergic agent. With the adrenals removed, however, there remains the possibility of liberation of sympathin at adrenergic nerve endings which in turn may stimulate the elaboration of a cholinergic substance. This sympathin, like hepatic sympathin, would have no mydriatic action (Cannon and Rosenblueth, 1935). Moreover, it is possible that the dilatation of the conjunctival vessels and the miosis of the denervated iris as noted are due to sympathetic nerve stimulation. Rosenblueth and Cannon (1935) postulated the theory that certain vasodilator effects in the cat are produced by adrenergic nerves.

Whatever mechanism may exist to explain these observations, it is evident that adrenergic and cholinergic humoral agents operate in insulin

hypoglycemia. The effects noted in the body are the result of interaction between the two substances. At times the adrenergic and less often the cholinergic effects seem to prevail.

SUMMARY

1. In the cat insulin hypoglycemia causes the sympathetically denervated pupil to dilate, the nictitating membrane to retract and the ear vessels to constrict, all indicative of the presence of a humoral adrenergic substance.

2. In the monkey insulin hypoglycemia produces dilatation of the sympathetically denervated pupil only after the administration of cocaine. In the absence of hypoglycemia, cocaine produces little or no dilatation of the s.d. pupil. This suggests the possibility of the discharge of an adrenergic substance.

3. Late in the course of insulin hypoglycemia, less conspicuous apparently cholinergic effects occur, consisting of slight contractions of the denervated face of either species. The completely denervated pupil of the hypoglycemic cat shows an initial dilatation and later a constriction, whereas the monkey shows no dilatation only a constriction. Bilateral adrenalectomy abolishes the dilatation and makes the constriction much more evident. This constriction of the pupil is suggestive of the presence of a cholinergic substance in hypoglycemia.

4. It is concluded that adrenergic and cholinergic humoral effects take place in response to hypoglycemia, the adrenergic phenomena predominating in the cat.

5. Attention is drawn to the much slighter autonomic effects demonstrable in the monkey. This may be explicable on the basis of relative insensitivity of the indicators rather than as significant of a fundamental difference in autonomic responses.

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ACUTE HYPOPROTEINEMIA FOLLOWING A SINGLE SEVERE HEMORRHAGE IN THE FASTING DOG[1]

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A severe hemorrhage is a serious and important event and one which frequently leads to the death of the patient often within a few hours. Important in the changes which follow hemorrhage is the protein content of the plasma because it is concerned with the maintenance of the osmotic pressure of the circulating blood. The protein lost in a severe bleeding is eventually restored, the process requiring a number of days, provided an adequate protein diet is ingested. There is considerable disagreement as to what happens within the first hours immediately following the hemorrhage particularly when no food has been ingested. In a preliminary communication (1) data were presented indicating that very little if any improvement in the acute hypoproteinemia occurred in the fasting state. Further and more detailed experimental findings are herewith presented.

Previous studies. Apparently the first study on the changes in serum protein following bleeding was reported in 1906 by Morawitz (2). This worker bled 7 dogs, replacing simultaneously an equal volume of a suspension of erythrocytes obtained from another dog. This procedure in 1914 was given the name of plasmapheresis by Abel, Rowntree and Turner (3). An analysis of the serum albumin figures presented by Morawitz shows that between the 3 and 18 hour specimen there was very little change, i.e., the averages are 2.6 and 2.7 grams per cent respectively. During the same period the serum protein rose from 3.4 to 3.9 grams per cent. A Kjeldahl method was used for determination of the proteins.

Beginning in 1918 appear the extensive studies of Kerr, Hurwitz and Whipple (4) on regeneration of blood serum proteins. They used the plasmapheresis technic, and although they fractionated their serum and determined the albumin separately, the refractometer was used in calculating the protein concentration. They found a very slow return of the depleted serum protein requiring many days. They state, "It seems that

[1] Aided by a grant from the Louis B. Beaumont Fund and by a grant from Mead Johnson and Company.

the body can produce serum protein only in small amounts even in an emergency." Two years later Smith, Belt and Whipple (5) reported further similar experiments on the curve of regeneration following plasmapheresis.

The curve of restoration of plasma protein following an almost complete plasma depletion was reported by Stanbury, Warweg and Amberson (6) in 1936. By replacing the blood with red cells suspended in acacia and saline they were able to avoid shock and yet achieve, by repeated bleeding and replacement, an almost total absence of plasma protein. They found that in the liberally fed dog replacement is complete in about 8 days. In 5 other dogs they noted a rise from a low total protein of 0.08 per cent to 1.01 per cent 4 hours later, which they attribute to the existence of a store of readily available serum protein.

Remarkably rapid return of total plasma protein following hemorrhage in rats was reported by Cutting and Cutter (7). They observed in 12 hours a complete restoration after 40 per cent of the calculated blood volume was bled. The animals were allowed water but no food during the experiment. No fractionation was reported.

Neumann (8) in rabbits found very little return of the low serum protein produced by repeated hemorrhages. From his observations he concluded that after hemorrhage blood volume was restored by the influx of a fluid which was poor in protein, rich in electrolyte. In two rabbits no increase in the serum protein concentration occurred even after 24 hours. He used a refractometric method for determination of serum protein.

EXPERIMENTAL PROCEDURE. The standard hemorrhage used equalled (in cubic centimeters) 3.5 per cent of the body weight (in grams) of the dog, selected from among healthy, adult, not too obese animals. Under local anesthesia the femoral artery was exposed, cannulated, and the calculated amount of blood removed. Immediately the same volume of warm Ringer's solution was replaced. In the early experiments this solution was injected into the adjacent femoral vein; later it was found more convenient and more rapid to replace the Ringer's solution directly into the artery through the same cannula and tube used in the bleeding. In this way the entire procedure of bleeding and replacement with Ringer's solution rarely required more than a few minutes, never over 5 minutes. Moreover, under these conditions severe shock rarely occurred and fatalities followed only on two occasions. These two cases were not included in the present data.

Samples of blood were obtained from the jugular vein before and immediately after the hemorrhage and replacement with Ringer's solution. Thereafter additional samples were removed in periods up to 6 hours, generally at 15 minutes, 30 minutes, 1 hour and 6 hours; in a few experiments 24 hour samples were also studied. All animals were fasted for at

least 48 hours preceding, and during the experiment though allowed water *ad lib.* except during the day of the experiment.

For determination of serum protein the blood was allowed to clot, centrifuged and the clear serum collected and analyzed for non-protein and total nitrogen. For the former Nessler's reagent was used, for the latter a titrimetric micro-Kjeldahl procedure, involving steam distillation of the ammonia into a boric acid solution (9). For the fractional determination of albumin Howe's method was employed. In later experiments a shorter modification was employed as described by Campbell and Hanna (10).

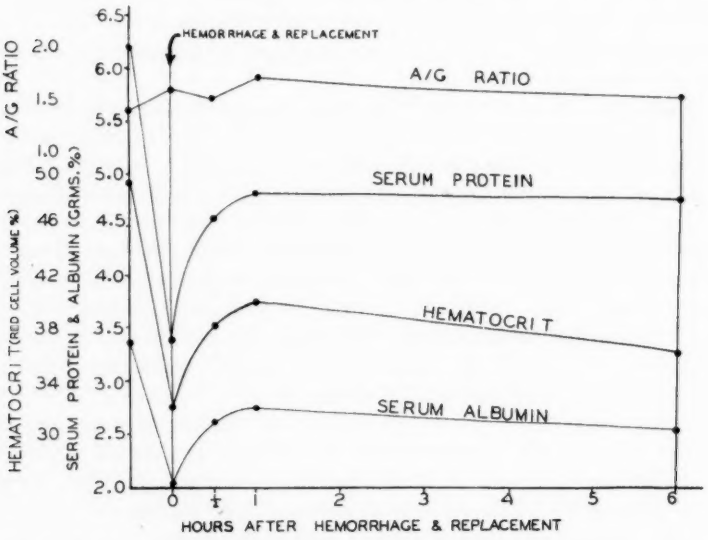

Fig. 1. Curves obtained by plotting the averages obtained from seventeen experiments. Note the absence of change in the serum protein and albumin after the 1 hour level is reached. Note also the drop in hematocrit between and the 1 and 6 hour values indicating a dilution and thus an increase in the total plasma volume. Note also the parallel nature of the curves and the relative constancies of the A/G ratio. For a discussion of the curve preceding the 1 hour value see text.

For the hematocrit a 5 cc. portion of blood was carefully introduced into a hematocrit tube containing 1 cc. of 1.6 per cent sodium oxalate, mixed and centrifuged at 3000 rate per minute for 30 minutes.

Experimental findings. The findings are summarized in figure 1. Following the bleeding and replacement there was a pronounced fall in serum protein, albumin, and red cell volume, each of which, however, rose sharply and reached a level within one hour. Thereafter the serum protein and albumin remained the same for the next 6 hours; in two dogs the 24 hour

sample showed no further change. The hematocrit, unlike the serum protein, fell significantly from the first to the sixth hour, indicating a dilution of the blood presumably by fluid entering the capillaries from the tissue spaces. The A/G ratio remained relatively unchanged throughout the experiment.

In all, 17 dogs are represented in the data herein presented. Though a complete range of determinations was not made in each experiment the number of observations used for averages was never under 11 in the case of the serum protein and hematocrit. In the case of serum albumin and A/G ratio the number of observations were smaller and thus less significant statistically.

COMMENT. It seems apparent from the present findings that after the first hour immediately following the hemorrhage and replacement there is little if any change for at least 6 hours in the concentration of the serum protein or of its two components, albumin and globulin. This does not mean that there is very little if any endogenous restoration of serum protein; the apparent increase in plasma volume suggested by the decreased hematocrit reading may indicate an actual increase in the total circulating plasma protein. Thus it would seem, following a severe hemorrhage under fasting conditions, the body is unable to increase significantly the serum protein concentration even though it may be able to add to its total amount. From the practical point of view the avoidance of shock after a severe hemorrhage depends upon the ability of the body to restore volume; yet the maintenance of the restored volume depends on its osmotic pressure which in turn is directly controlled by the concentration of serum protein, especially its albumin fraction. Inasmuch as the body seems unable to increase the concentration of serum protein rapidly, it would seem that therapeutic efforts might well be directed along this direction.

The changes in the serum protein during the first hour following hemorrhage and replacement demand some explanation. At first sight the spectacular increase might be interpreted as due to the entrance of a ready source of protein from some body store. While this may be true, a more direct explanation may be offered. The parallel curve in the hematocrit readings as well as the relative constancy of the A/G ratio suggest a purely physical cause for the great though short lasting increase in serum protein concentration. During the period of bleeding, short as it may be, protein-poor tissue fluids are undoubtedly drawn into the blood stream to make up for the lost blood; this is a normal, prompt, and well-known reaction. Into the blood stream is then injected an additional volume of protein-free fluid equal in amount to that withdrawn. Thus, at the end of replacement the blood volume must be greater than at the beginning and the serum protein concentration correspondingly less. Immediately, however, the superfluous fluid begins to leave the blood stream and enter

the tissue spaces thus lowering the blood volume and leading to a corresponding increase of the concentration of serum protein. The sharp fall and rise could thus be explained by the rapid changes in the plasma volume induced by the experimental manipulation. The hypoproteinemia reached in 1 hour would seem to be the true end result of the loss of serum protein in the removed blood. It is hard to see why an actual increase of serum protein by regeneration (or by any other mechanism) would be so rapid for one hour and then suddenly cease. It should be stated that fibrinogen is not considered in this study; there is excellent evidence that this fraction of the plasma proteins can be manufactured (or enter the blood as such) very rapidly.

It will be noted also that the average drop (at one hour) in the serum protein was 1.4 grams per cent, in the serum albumin 0.63 gram per cent, or, as a percentage of the pre-hemorrhage level, of 22 and 20 per cent respectively. If one assumes that the dog's blood volume equals 12 per cent of the body weight the 3.5 per cent removed would approximate 29 per cent of the total blood. The actual fall in serum protein is but 9 per cent less thus indicating but a slight increase in the serum protein concentration over the theoretical level as calculated without the assumption of any restoration from "body stores." The findings herein reported do not, therefore, support the idea of a significant, readily available store of "body protein" which is immediately available to restore lost serum protein after hemorrhage.

SUMMARY

1. Acute hypoproteinemia averaging a drop of 1.4 grams per cent in the concentration of serum protein and 0.63 gram per cent in the serum albumin was produced in fasting dogs within one hour after an arterial bleeding amounting to 3.5 per cent of the body weight, with immediate replacement of the same volume of Ringer's solution. The fall in serum protein and serum albumin averaged 22 and 20 per cent respectively of the pre-hemorrhage level.

2. The hypoproteinemia thus produced did not change significantly for 6 hours and perhaps longer; there was some evidence, however, of an increase in total circulating serum protein during this period.

3. These findings indicate that there is no rapid mechanism for the restoration of the concentration of serum protein or serum albumin lost by hemorrhage in the fasting dog, and emphasize the importance of inducing such a restoration therapeutically in patients suffering severe blood loss.

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EFFECT OF EXERCISE ON THE BLOOD FLOW IN THE SUPERIOR MESENTERIC, RENAL AND COMMON ILIAC ARTERIES

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When the blood flow to a given region of the vascular bed is increased markedly, it has been generally assumed that the blood flow to other regions is decreased. During exercise the blood flow to the musculature is greatly increased so that one would expect to find a concomitant decrease elsewhere. Because there is evidence that constriction of certain vessels of the abdominal viscera occurs during exercise, it is assumed that the flow through these vessels is decreased. However, one is not justified in making this deduction. The term constriction does not necessarily connote decreased volume flow. When the constriction is moderate, the increased peripheral resistance may be counterbalanced by an increased blood pressure so that no decrease in blood supply occurs. The increase in pressure may bring about an increase in blood velocity in the constricted vessels so that the total volume flow (velocity $\times$ cross-sectional area) remains constant. In fact, there may be an actual increase in flow under such conditions.

METHODS. The thermostromuhr method of measuring blood flow permits observation during exercise. The blood flow unit is sterilized and aseptically applied to the desired vessel of a dog under ether anesthesia. When the dog recovers from the operation blood flow observations may be made for a period of two weeks or more. During this time the dog appears in good condition. It eats well, runs about freely, exercises on the treadmill and behaves normally.

In this particular study dogs weighing from 12 to 22 kgm. were trained to walk or run on the treadmill and to lie on it quietly when not in motion. Thermostromuhr units were applied to the superior mesenteric and femoral arteries or to the renal and femoral arteries of the same dog. After a few preliminary experiments were performed it was decided to apply the unit to the common iliac instead of the femoral artery.

A midline abdominal incision extending from the xiphoid process to a point midway between umbilicus and symphysis pubis was employed in

female dogs. In male dogs the skin incision did not extend so far caudally; the same degree of exposure of the left common iliac artery was obtained by splitting the lower part of the left rectus longitudinally in such fashion that most of the muscle could be retracted laterally, allowing a left paramedian incision of the posterior rectus sheath and peritoneum.

The superior mesenteric artery was exposed close to its point of origin by lateral retraction of the peritoneum of the root of the mesentery, and of the superior mesenteric ganglion. The left renal artery likewise was exposed near its origin from the aorta. In the case of both of these vessels the region near the origin is the only site sufficiently free of branches to permit of application of the thermostromuhr. An especially devised retractor, the blade of which was formed to the shape of the medial aspect of the kidney, facilitated laterocaudal retraction of the kidney and made easy the dissection of the renal artery while it was under tension.

The vessel finally used for the measurement of blood flow to the left hind extremity was the left common iliac artery. The application of the thermostromuhr unit to the femoral artery, while practical in a few instances, was too often attended by wound infection involving the femoral artery and causing thrombosis. The external iliac artery was unsatisfactory because of early thrombosis, collateral circulation from the hypogastric artery being readily established.

The thermostromuhr units chosen for application to the vessels to be measured were of such size as to fit the vessels snugly and cause slight constriction. A unit having an internal diameter of 2.5 mm. was usually used for the left renal artery, of 3.0 mm. for the superior mesenteric and femoral arteries, and of 3.5 mm. for the left common iliac artery. The superior mesenteric and renal arteries are intimately surrounded by sympathetic nerve fibers and the gentle yet complete dissociation of the vessels, for a distance of three-fourths inch (2 cm.), from these fibers and other tissue constituted the major part of the operation. Traction on the superior mesenteric ganglion during the freeing of the superior mesenteric artery produced mild nausea and vomiting for the next twenty-four hours.

The lead wires from the left common iliac and left renal arteries, or left common iliac and superior mesenteric arteries, were loosely anchored to peritoneum in the dorsolateral abdominal wall adjacent to the units and then led through separate stab wounds through the muscular portion of the anterior abdominal wall. Tiny portions of omentum were wrapped around the intra-abdominal portion of the lead wires and fastened to the stab wounds. From the muscle wall the lead wires were led subcutaneously a short distance and finally passed through skin stab wounds.

In all experiments the treadmill was operated at the same speed, that is, 3 miles per hour. The duration of exercise varied from ten to twenty minutes.

RESULTS. In each experiment the flow in the femoral artery or in the common iliac artery increased. Usually the increase was from two to ten times the flow at rest. The increase took place immediately and the photographic records were similar to those reported from this laboratory on the effect of exercise on the flow in the coronary artery (left circumflex branch) (10). Increasing the amount of exercise by elevating the treadmill about 15 degrees usually caused an additional increase in flow. For the most part, the flows approximated 1000 cc. per minute or even higher while the treadmill was elevated. This necessitated calibrating the blood flow units up to this range. The linear relation (on logarithmic graph paper) between the deflection of the galvanometer in the thermocouple circuit and the flow of blood in cubic centimeters per minute persisted. The range of flows in our calibrations was from about 50 cc. per minute to 1200 cc. per minute. This linear relation indicates that the flows in this range are not turbulent. Some flows exceeded our range of calibration so that we cannot deny the possibility of turbulence in these cases. Nevertheless, our results are in agreement with the observations of others (10) regarding the effect of exercise on the blood flow in those vessels which supply, to a large extent, the muscles. Figures 1 and 2 (lower record) are representative of the results obtained.

Superior mesenteric artery. Seventeen experiments were performed on three dogs. Six experiments showed no significant change in flow during exercise. Ten showed significant increases ranging for the most part from 16 to 45 per cent. One of these ten showed an increase of 93 per cent. Only one experiment showed a decrease and that was 14 per cent. Changes in flow beyond 10 per cent are regarded as significant. After the treadmill was stopped the flow in some experiments (six of the seventeen) showed significant decreases. These decreases ranged from 14 to 32 per cent and averaged 23 per cent. All of these decreases with the exception of one occurred in one dog. Figure 1 shows a representative experiment.

Renal artery. Twenty-six experiments were performed on five dogs. Eleven experiments showed a definite increase in flow ranging from 18 to 76 per cent except for one experiment which showed a larger increase. Eleven experiments showed no significant change in flow during the period of exercise. Four experiments showed the following decreases: 18 per cent, 19 per cent, 22 per cent and 33 per cent at the beginning of exercise. Three of these observations were on one dog whose renal flows were low. Other observations on this same dog showed no significant change or a slight increase. This particular dog always appeared excited at the starting of the treadmill and never seemed to become accustomed to the persons present. The fourth decrease occurred during the first experiment following operation. The subsequent five experiments on the

same dog showed either an increase (as high as 88 per cent) or no significant change. Five experiments showed a decrease immediately following the period of exercise. These decreases were 14 per cent, 19 per cent, 23 per

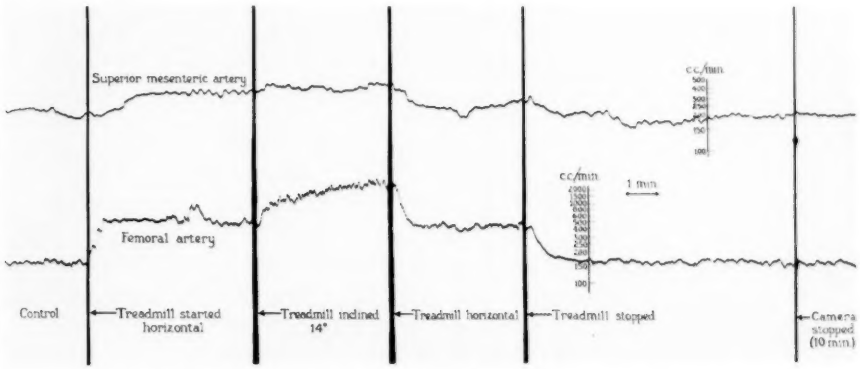

Fig. 1. The effect of exercise on the blood flow in the superior mesenteric artery and the femoral artery.

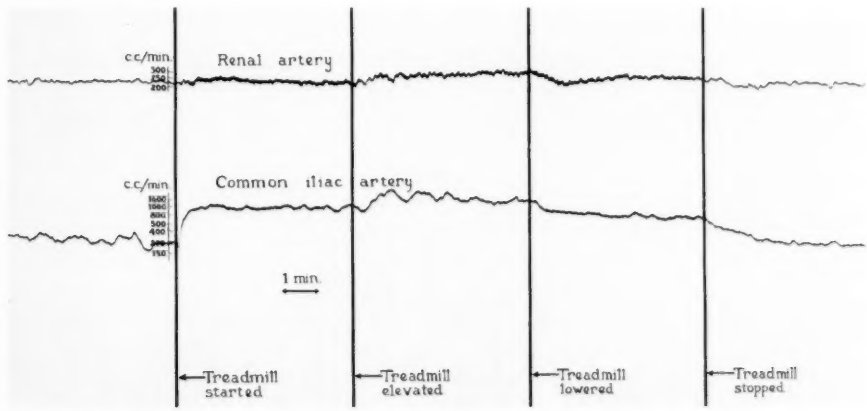

Fig. 2. The effect of exercise on the blood flow in the renal artery and the common iliac artery.

cent and 27 per cent respectively. Figure 2 shows a representative experiment.

Splenic artery. The results of the effect of exercise on the blood flow of the spleen appear in a separate study of the spleen (5). Observations were made in either the splenic artery or the splenic vein. A definite

increase in blood flow occurred in spite of the contracted state of the spleen reported by Barcroft and Florey.

The study by Barcroft and Loughridge on the accuracy of the thermostromuhr method for measuring blood flow might make one wonder about the possibility of changes in the temperature of the blood (caused by exercise) affecting the reliability of the blood flow data. For this reason we recorded the changes in the deflection of the galvanometer (with no heating current present) during exercise. In other words, we looked for variations in the zero of the galvanometer during exercise. Either no change or an insignificant change in the zero of the galvanometer during the period of exercise was noted over a period of three minutes. When one considers that the flow in the femoral or iliac artery changes immediately, he may feel confident that, whatever changes in blood temperature take place, they do not seem large enough to affect the values of blood flow in this particular experiment. We have observed deflections of the galvanometer when blood of a different temperature is added to the blood reservoir of our perfusion system during the calibration of units.

COMMENT. If one consults the literature regarding the effect of exercise on the blood flow to certain visceral organs, he is left with the impression that there is a decrease in blood flow. The assumed decrease in blood flow is based on the following indirect evidence: 1, increased blood pressure (caused by constriction of vessels of abdominal viscera); 2, contracted spleen; 3, decreased activity of the kidneys, or 4, pallor in an exteriorized patch of colon.

Increased blood pressure. The increase in blood pressure (an average of 36 per cent according to a previous study in our laboratory) (4) is probably due to constriction of the visceral vessels of the abdomen because the other factors contributing to elevated blood pressure do not seem sufficient. Although the cardiac output is increased markedly one cannot assume that this condition brings about the observed increase in blood pressure because the blood flow to the musculature is increased greatly. The increased flow to the musculature might counterbalance the increase in cardiac output so as to effect no significant change in blood pressure. The remaining factors which contribute to the maintenance of blood pressure, that is, blood volume, blood viscosity and elasticity of the blood vessels, seem to play a minor rôle toward elevating the blood pressure during exercise. The contracted spleen is cited as direct evidence of visceral constriction during exercise.

Contracted spleen. A separate study (made in our laboratory) (5) of the effect of exercise on the blood flow of the spleen showed that the flow is not decreased. Barcroft and Florey reported that the spleen contracts at the onset of exercise and remains contracted throughout the period of exercise. Evidently a contracted spleen does not indicate that the blood

flowing through it is necessarily decreased. The anatomic study of the circulatory system of living, unstimulated mammalian spleens, by Knisely, described the presence of shunts between the arterial and venous systems, independent of the sinusoids. This mechanism explains the possibility of a contracted spleen with no accompanying decrease in blood flowing through it. This is an instance where the method of plethysmography would fail to indicate the flow in, say, the splenic artery.

Suspension of activity of the kidneys. The inhibition of water diuresis during exercise is well established, as well as a decrease in urine output in the individual or animal that has not been given a diuretic. MacKeith, Pembrey, Spurrell, Warner and Westlake have published a detailed study of oliguria and anuria during muscular work in man. They attributed this condition to constrictor impulses sent to the splanchnic region in order to increase the volume of blood circulating through the muscles. They also explained the presence of albumin in the urine as a sign of the previous want of arterial blood in the kidneys. Other investigators agree with these authors regarding this causative factor of decreased kidney function during exercise.

The paper on inhibition of water diuresis by Rydin and Verney states that "The course of the inhibitory response to emotional stress is unaltered by successive operations comprising *a*, division of the renal nerves; *b*, removal of the right suprarenal and denervation of the left; *c*, decentralisation of the whole abdominal sympathetic system with removal of ganglia L2 to S1 inclusive." If one reflects on the vascular anatomy of the kidney with its two sets of capillaries he is not justified in assuming that decreased urine formation indicates necessarily a decreased blood flow through the kidneys. Walker, Schmidt, Elsom and Johnston have shown that changes in renal flow cannot be held responsible for the alteration in urine volume produced by water. We also have failed to find a parallelism between changes in urine flow and changes in blood flow to the kidney except in a few instances.

With regard to urinary constituents, little has been published on the relation of these to blood flow. For the dog Medes and Herrick found a parallelism between creatinine clearance and blood flow. Some investigators (3, 7) have reported an increase in creatinine excretion during exercise.

The possibility of arteriovenous shunts in the kidney should not be excluded when one wishes to interpret the results of our experiments. Such shunts have been demonstrated. Under these circumstances the blood flow in the renal artery is not an index of the flow through renal tissue.

Pallor in an exteriorized patch of colon. Barcroft and Florey found that the pallor of the colon was observed only in short bursts of running. Longer periods of exercise showed less pallor. In fact they are of the

opinion that the pallor may be due to excitement rather than to exercise per se. We have not observed the effect of exercise on the blood flow to the colon. However, one may assume that an exteriorized patch of colon is not representative of the abdominal viscera. Since the observed pallor is of short duration, it is a minor factor in the present study.

CONCLUSIONS

The blood flow in the common iliac artery is increased markedly during exercise. The blood flow in the superior mesenteric and renal arteries is not decreased during exercise. With few exceptions, there was either no significant change or a definite increase in the blood flow in the renal artery and the superior mesenteric artery while the dog was running on the treadmill (either horizontal or elevated about 15 degrees) at the rate of 3 miles per hour for a period ranging from ten to twenty minutes. In a separate study, exercise brought about no decrease in the flow in the splenic artery or in the splenic vein. Our observations allow vasoconstriction in these particular vessels because the increased peripheral resistance may be counter-balanced by the increased blood pressure so as to effect no significant change in volume flow or an actual increase.

The indirect evidence which led investigators to conclude that exercise brings about a decrease of the blood flow to the kidney and the spleen as well as of that in the superior mesenteric artery is discussed.

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A STATISTICAL STUDY OF COBALT POLYCYTHEMIA IN THE DOG

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In a previous communication (1) it was presumed that the failure to obtain a definite polycythemic response to cobalt in the dog was due to inadequate dosage; it was suggested that a polycythemia could be produced in the dog, if sufficient cobalt were given, because all other species of animal tested have shown this response. The amount at first used in those experiments (1) was approximately that (0.8 mgm. per kilo per day) claimed by Mascherpa (2) to produce a polycythemia. This dosage failing, the amount was increased to 4.0 mgm. per kilo per day. This dosage also seemed inadequate over a period of 26 days.

Subsequently, Davis (3) in two series of experiments, administered cobalt to four dogs in the amount of 2 mgm. per kilo per day and found what he interpreted to be a significant increase in the erythrocyte number. No claim for a coincident increase in hemoglobin percentage is made in his experiments. Because of this conflict of data it was concluded that the only rational approach for deciding the matter was a somewhat modified repetition of the experiment with statistical analysis of the results. Accordingly, this has been done.

METHODS. The care of animals and the technique of blood counting was that previously described (1). The method of blood sampling was modified in that 2 ml. were drawn from the jugular vein into the oxalate mixture suggested by Wintrobe (4). In addition to the erythrocyte count, hemoglobin determinations were made by the Newcomer plate method and the percentage volume of packed red cells determined by means of the Wintrobe hematocrit tube (5).

Five young healthy adult female dogs, well conditioned to laboratory handling, were used. Cobaltous chloride was mixed in the food in varying amounts equivalent to 5, 10, 15 and 20 and 30 mgm. of cobalt per kilo per day. At no time during the course of the four week period of cobalt feeding were any toxic symptoms noted. This was the case also for two dogs who received 50 mgm. per kilo per day for a period of two weeks.

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The statistical management of the data is one described by Fisher (6) which depends upon a calculation of the standard deviations (s) of the difference of the means ($\bar{x}_1 - \bar{x}_2$) of the series before and after cobalt administration. From a table of the distribution of $t \left(= \frac{(\bar{x}_1 - \bar{x}_2)}{s} \right)$, P is determined. No significance can be attributed to differences whose value of P exceeds 0.05.

TABLE 1

The blood before and after cobalt administration

	ANIMAL	NUMBER OF MEASUREMENTS BEFORE COBALT	MEAN ($\bar{x}_1$)	AMOUNT OF CO	NUMBER OF MEASUREMENTS DURING COBALT	MEAN ($\bar{x}_2$)	DIFFERENCES OF MEANS ($\bar{x}_1 - \bar{x}_2$)	s ($\bar{x}_1 - \bar{x}_2$)	$t \frac{\bar{x}_1 - \bar{x}_2}{s}$	P
				mgm. per kilo per day						
R.B.C. in millions per cu. mm.	A	9	6.25	5	6	6.86	+0.61	0.286	2.13	0.1–0.05
	B	11	6.06	10	7	6.90	+0.84	0.411	2.04	0.1–0.05
	C	13	5.70	15	6	6.76	+1.06	0.356	2.98	0.01
	D	7	5.97	20	6	6.86	+0.89	0.390	2.28	0.05–0.02
	E	9	6.09	30	7	7.12	+1.03	0.184	5.60	0.01
Hemoglobin in grams (Newcomer plate)	A	9	16.8	5	6	17.3	+0.5	0.751	0.665	0.6–0.5
	B	10	15.8	10	7	17.9	+2.1	0.560	3.75	0.01
	C	10	15.1	15	6	16.8	+1.7	0.605	2.81	0.02–0.01
	D	7	15.9	20	7	18.0	+2.1	0.680	3.09	0.01
	E	9	16.4	30	7	19.1	+2.7	0.730	3.70	0.01
Percentage volume packed red cells	A	9	44.4	5	6	45.3	+0.9	1.59	0.567	0.6–0.5
	B	11	43.8	10	7	46.7	+2.9	1.60	1.81	0.1–0.05
	C	13	42.4	15	6	44.0	+1.6	1.54	1.04	0.4–0.3
	D	7	44.2	20	7	47.6	+3.4	1.31	2.59	0.05–0.02
	E	9	43.3	30	7	49.1	+5.8	1.34	4.33	0.01

RESULTS. In table 1 the data and their statistics are summarized. It may be seen at once that dog A (5 mgm. Co) showed no significant change in erythrocyte count, amount of hemoglobin or hematocrit findings. Dog B (10 mgm. Co) similarly showed no significant increase in erythrocyte count or in hematocrit reading but did show a significant increase in hemoglobin. Dog C (15 mgm. Co) showed an increase in erythrocyte count and in hemoglobin but none in percentage volume of packed red cells. Dogs D (20 mgm. Co) and E (30 mgm. Co), on the other hand, both showed unequivocal increases in erythrocyte count, hemoglobin and hematocrit.

In view of these results it was thought advisable to review statistically in a similar fashion the author's previous data (1) as well as those of Davis (3). These analyses are embodied in tables 2 and 3.

TABLE 2

The R.B.C. number in millions before and during administration of 4 mgm. of cobalt per kilo per day (from previous data (1))

ANIMAL	NUMBER OF MEASUREMENTS BEFORE COBALT	MEAN ($\bar{x}_1$)	NUMBER OF MEASUREMENTS DURING COBALT FEEDING	MEAN ($\bar{x}_2$)	DIFFERENCE OF MEANS ($\bar{x}_1 - \bar{x}_2$)	STANDARD DEVIATION OF DIFFERENCE OF MEANS $s(\bar{x}_1 - \bar{x}_2)$	$t \frac{(\bar{x}_1 - \bar{x}_2)}{s}$	P
H	4	5.23	9	5.43	+0.20	0.0194	10.3	0.01
A	7	5.25	9	5.29	+0.04	0.0298	1.34	0.2
B	7	6.52	9	6.64	+0.12	0.1109	1.08	0.3-0.2
L	7	6.70	9	7.04	+0.34	0.1233	2.75	0.02-0.01

TABLE 3

The blood before and after administration of 2 mgm. of cobalt per kilo per day (from data of Davis (3))

	ANIMAL	NUMBER OF MEASUREMENTS BEFORE COBALT	MEAN ($\bar{x}_1$)	NUMBER OF MEASUREMENTS DURING COBALT	MEAN ($\bar{x}_2$)	DIFFERENCE OF MEANS ($\bar{x}_1 - \bar{x}_2$)	$s(\bar{x}_1 - \bar{x}_2)$	$t \frac{\bar{x}_1 - \bar{x}_2}{s}$	P
R.B.C. in millions per cu. mm.	1(a)	7	4.85	8	5.11	+0.26	0.0398	6.53	0.01
	1(b)	7	4.85	5	5.04	+0.19	0.0298	6.37	0.01
	2(a)	7	5.81	7	5.91	+0.10	0.110	0.91	0.4-0.3
	2(b)	7	5.81	5	5.62	−0.29	0.0269	7.06	0.01*
	6(a)	6	5.16	8	5.88	+0.72	0.271	2.65	0.02
	6(b)	6	5.16	5	5.71	+0.55	0.182	3.02	0.02-0.01
	7(a)	5	5.19	7	5.84	+0.65	0.141	4.64	0.01
	7(b)	5	5.19	6	5.45	+0.26	0.226	1.15	0.3-0.2
Hemoglobin in per cent (Sohli)	1(a)	7	69	8	71	+2	0.850	2.35	0.05-0.02
	1(b)	7	69	5	73	+4	0.672	5.95	0.01
	2(a)	7	84	7	79	−5	1.83	2.74	0.02-0.01*
	2(b)	7	84	5	73	−11	2.54	4.31	0.01*
	6(a)	6	71	8	76	+5	1.67	2.99	0.02-0.01
	6(b)	6	71	5	82	+11	0.94	11.7	0.01
	7(a)	5	70.2	7	70.4	+0.2	1.18	0.17	0.9-0.8
	7(b)	5	70.2	6	72	+2	0.925	2.16	0.1-0.05

* Statistically significant *decreases*.

From table 2 it is to be seen that administration of 4 mgm. of cobalt did actually cause a significant increase in red cell number in 2 of 4 dogs. It may be remarked, however, that in one case (dog H) only 4 control ob-

servations were made, apparently a number insufficient to give an accurate picture of the magnitude of daily normal variation. Dog L on the other hand unquestionably experienced an increase in erythrocyte number. Dogs A and B show no significant increases during cobalt administration.

From table 3 we see that both experiments (a and b) on dog 1 result in significant increases in the erythrocyte count. In the first experiment on dog 2 (a) there is no significant increase in the erythrocyte count while in the second experiment (b) there is actually a statistically significant *decrease* in the red cell count during cobalt administration. Dog 6 in both experiments (a and b) responds by what is a significant increase in the erythrocyte count. Dog 7 shows a significant increase in red cells in the first experiment (a) but not in the second (b).

The hemoglobin data of Davis (3) are remarkable in that only three of the experiments show a significant increase while three show actual *decreases* in hemoglobin percentage following cobalt administration and two of these are statistically significant.

A similar analysis of the data of Mascherpa is not feasible as his experiments were uncontrolled by a series of preliminary observations.

SUMMARY

From the analyses it seems rather conclusive that cobalt in doses of 2 mgm. per kilo per day causes no regular and predictable increase in the erythrocyte number. The hemoglobin is as frequently decreased as increased. The author's data show no regular significant increase in the erythrocyte number until the daily dose of cobalt exceeds 10 mgm. per kilo. Similarly, the hemoglobin concentration is increased only by doses in excess of 5 mgm. while the percentage volume of packed red cells is increased by cobalt only when the amount fed daily exceeds 15 mgm. per kilo.

It may be concluded that cobalt in sufficient dosage causes a significant increase in erythrocyte number, hemoglobin concentration and percentage volume of packed erythrocytes in the dog without toxic symptoms appearing. Five milligrams of cobalt per kilo of body weight seems to be close to the critical concentration required to increase the erythrocyte number and hemoglobin concentration whereas the hematocrit is increased only by somewhat larger doses.

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THE EFFECT OF WARM AND OF COLD NASOPHARYNGEAL IRRIGATION ON CERVICAL LYMPH FLOW[1]

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Modifications in lymph flow due to local applications of heat have been reported in the literature from time to time. Starling, in 1894, stated that an increase in lymph flow and a higher lymph protein concentration occurred when water at 60°C. was applied to the foot of a dog for 5 minutes. Field, Drinker, and White (1932) placed an anesthetized dog's foot in a water bath and gradually raised the temperature of the bath to 60°C. They found no marked increase in lymph flow below 50°C. Between 50° and 60°C. the flow became profuse and the lymph contained a higher percentage of protein. However, Beazell, Schmidt, and Ivy (1937), working on dogs, report no change in lymph flow from mesenteric lymphatics when water of 52.2° to 57.7°C. was circulated through a rubber applicator placed in an intestinal loop, although the blood flow in this region was doubled or even quadrupled.

A preparation (McCarrell, 1939) which produces a constant cervical lymph flow in an anesthetized dog and at the same time permits warm or cold saline irrigation of the nasopharynx has been utilized in the present study of this problem. Certain critical temperatures, at which cervical lymph flow is altered, were determined in a series of 8 dogs.

METHOD. Dogs were anesthetized by an intraperitoneal injection of nembutal (40 mgm. per kilogram of body weight), and the right and left cervical lymph vessels were cannulated. The snout of the dog was attached to an electrically driven crank which, as it rotated, slowly flexed and extended the neck. This passive motion insured a constant flow of lymph without the use of massage. Lymph from the two cervical vessels was collected, pooled, weighed, and the flow calculated in milligrams per minute. Protein concentration was determined refractometrically. The effect on lymph flow of the local application of heat or of cold was de-

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termined by irrigation of the nasopharynx with isotonic saline at temperatures ranging from 5° to 55°C., the same temperature being continued for a period of either 20 or 30 minutes. The body temperature was maintained within the normal range by means of ice packs or heat applied to the surface of the body when necessary. Sections of nasopharyngeal mucosa were removed at the conclusion of each experiment for histological examination. The details of these procedures appeared in a previous paper (McCarrell, 1939).

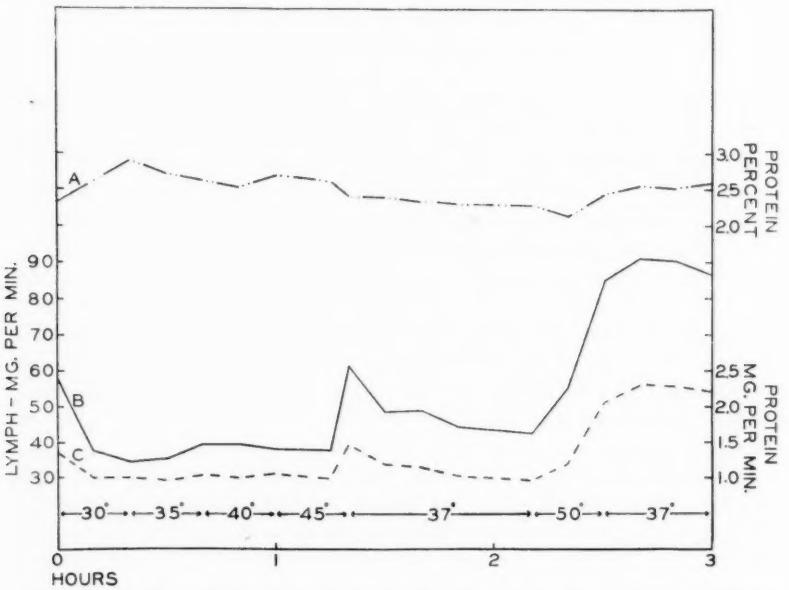

Fig. 1. The effect of nasopharyngeal irrigation with warm saline on cervical lymph flow and protein content. Lymph from right and left vessels pooled. *A*, protein per cent; *B*, lymph flow in milligrams per minute; *C*, protein in milligrams per minute. Temperatures of irrigating fluid in degrees centigrade.

RESULTS. 1. *Heat.* Nasopharyngeal irrigation with isotonic saline at 37°C. had no effect on either cervical lymph flow or its protein content. The actual amount of lymph produced by any one animal showed considerable variation, the values ranging from 8.7 to 63.5 mgm. per minute. Increasing the temperature up to 44°C. also caused no significant changes in the lymph. At 45°C., however, the production of lymph usually increased. In a few cases this increase was not observed or was very slight, but in the majority of the experiments the flow was almost doubled. A

lag of 10 to 15 minutes occurred before a significant increase was apparent. The change was a temporary one, the flow returning to a lower level soon after the temperature was reduced to 37°C. Alterations in the amount of lymph protein, calculated in milligrams per minute, accompanied the changes in flow. The protein percentage remained about the same or decreased slightly, indicating that capillary permeability with regard to plasma proteins had not increased. Histological sections of the nasopharyngeal mucosa showed some hyperemia and slightly enlarged lymphatics, but no evidence of tissue damage.

When the temperature of the irrigating fluid was raised to 50°C. or above, the lymph flow increased tremendously. In two instances values approximately three times that of the normal were obtained. This lymph was highly proteinized. These changes were irreversible and the values did not return to their former levels when the temperature was reduced to 37°C. Microscopic sections of the mucosa showed evidences of tissue damage as well as hyperemia and swollen lymphatics.

Figure 1, from results obtained during one experiment, illustrates the typical effects of temperatures of 45° and 50°C. A 20-minute period at 45°C. increased the lymph flow from an average value of 35.7 to a value of 61.2 mgm. per minute. This increase was not due to capillary damage, as lymph production decreased when the temperature was reduced to 37°C. A subsequent 20-minute period of irrigation at 50°C. caused the flow to rise from a level of 43 mgm. to that of 91 mgm. per minute. The protein percentage was higher, and the protein in milligrams per minute rose from 0.97 to 2.32. These values remained at a high level even when the temperature of the irrigating fluid was reduced to 37°C. A definite and sustained increase in capillary permeability must have taken place as a result of the heating.

2. *Cold.* Reducing the temperature of the irrigating fluid below 37°C. caused no marked changes until a temperature of 5°C. was reached. At this point, after a latent period of 10 to 15 minutes, the flow became definitely greater. This increase was either temporary, lasting only for the duration of the cold irrigation, or was more prolonged. This is shown in figure 2. The first period of irrigation at 5°C. caused, after a latent period of 10 minutes, a temporary rise in lymph flow with no change in the percentage of protein. When the temperature was increased to 37°C. the flow decreased and the protein percentage dropped to its lowest level in this particular experiment. The second irrigation with cold saline caused a more sustained increase in lymph flow with a rise in the percentage of protein. Histological examination of the nasopharyngeal mucosa showed hyperemia as well as epithelial damage in some regions. At 5°C., unlike the situation found at 45°C., normal physiological adjust-

ments, leading to a temporary augmentation of lymph flow, may be succeeded by capillary damage and a prolonged flow of more highly proteinized lymph.

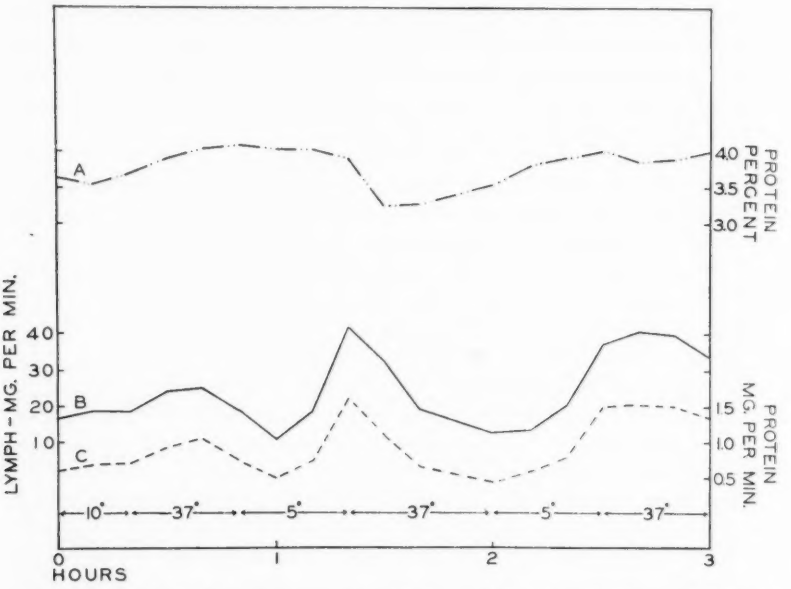

Fig. 2. The effect of nasopharyngeal irrigation with cold saline on cervical lymph flow and protein content. Lymph from left cervical vessel only. *A*, protein per cent; *B*, lymph flow in milligrams per minute; *C*, protein in milligrams per minute. Temperature of irrigating fluid in degrees centigrade.

Discussion. The alterations in lymph flow brought about by certain critical temperatures are the result of the direct effect of heat or cold on the local blood vascular bed. Increased filtration of intracapillary fluid, brought about by temperature changes, may be due to one or more of the following factors (Landis, 1934): *a*, dilatation of capillaries or opening of those previously closed; *b*, a rise in intracapillary pressure, and *c*, direct injury to capillary walls, making them abnormally permeable to plasma proteins. These factors and the temperatures at which they are brought into play have been investigated in various ways.

Lewis (1927) found that placing the arm in water at 43° to 45°C. caused a local reddening of the skin which was due to dilatation of the small vessels in and under the skin. At 51° to 52°C. wheals and blisters, evidences of local injury, were produced. Lewis also states that a temperature of 0° or 5°C. always reddens the hands of healthy people. This,

likewise, is caused by dilatation of the minute vessels, and continues until the cold (−2°C.) freezes and damages the tissue. Lake (1917) reported that for human skin and rabbit-ear skin little or no damage was done by exposure to cold until −6°C. was reached and the tissue was solidified. Hudack and McMaster (1932), using their vital dye technique in the mouse ear, reported that a 10-minute application of water at 1°to4°C. caused active hyperemia and a more rapid escape of dye from the capillaries. The same was true for a temperature of 44°C. for 14 minutes. Water at 45°C. for 14 minutes, according to these investigators, injured the vessel walls as was shown by the loss of the dye permeability gradient and the presence of extensive edema. Landis (1934) raised the skin temperature of the finger to 42°C. and found that the capillary pressure increased from values of 32 and 12 mm. Hg in the arteriolar and venous segments, respectively, to values of 60 and 45 mm. Hg. According to Landis, "the excess of capillary blood pressure over the colloid osmotic pressure of the blood must lead to the filtration of fluid which presumably passes on into the lymphatic vessels."

The majority of the evidence seems to indicate that at 0° to 5°C. and at 42 to 45°C. local applications of cold or heat to the surface of the body cause an increase in the amount of fluid filtering from the capillaries into the tissue spaces, and that this is brought about by a rise in intracapillary pressure and by an increase in the filtration area due to capillary dilatation. Below 0°C. and at about 50°C., however, the capillary walls are damaged, become extremely permeable to plasma proteins, and allow the escape of a highly proteinized fluid.

Beazell, Schmidt, and Ivy (1937) applied heat to isolated segments of intestine by means of a rubber applicator through which water circulated. They report that temperatures of 52.2° to 57.7°C. increased the blood flow but had no measurable effect on lacteal lymph flow unless the applicator, by distending the segment and thus reducing the blood supply and the dissipation of heat, caused tissue damage. Lymph flow was measured in cubic centimeters per hour, and it is possible that small variations in the scanty lymph flow from the intestinal loops were not detected.

SUMMARY

Cervical lymph flow was measured quantitatively in dogs during 20 or 30-minute periods of nasopharyngeal irrigation with isotonic saline at temperatures ranging from 5°C. to 55°C. Passive motion of the head of the animal insured a constant lymph flow without the use of massage. Critical temperatures, with respect to lymph flow and protein content, were found to correspond closely to those reported for general vascular changes influencing capillary filtration.

Continuous irrigation with isotonic saline at temperatures above 5°C.

and below 45°C. caused no marked variations in lymph flow or in its protein content. At 45°C. the lymph flow increased with no change in the protein percentage. This augmented flow continued only during the period of heat application and was the result of a rise in the rate of capillary filtration. At 50°C. and above, irreversible changes in capillary permeability occurred. The lymph flow became profuse, was highly proteinized, and did not return to normal with reduction of the temperature. Irrigation at 5°C. produced an augmented lymph flow which, depending upon the absence or presence of capillary damage, was either temporary or more prolonged.

The writer wishes to thank Dr. Cecil K. Drinker for suggesting this problem and for his interest and help during its course.

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THE INFLUENCE OF REPRODUCTIVE CONDITION UPON GROWTH IN THE FEMALE RAT[1]

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Considerable attention has been paid during the past few years to the influence of diet upon growth and we now have a fairly good idea of the nature of such factors both upon the rate of growth and upon the ultimate size which may be reached. But very scanty data are available upon the nature of the changes in growth which are produced by differences in the reproductive regime, yet such information has not only biological but also sociological and practical value.

Slonaker (1927), the pioneer worker in this field, compared the rate of growth of rats kept as virgins, those normally bred and allowed to lactate, and those normally bred but not allowed to lactate. However, he subdivided his groups into exercise and no-exercise divisions, and the virgins still further into an hysterectomized group. As a result his numbers are small for individual groups. His main conclusions which bear upon the problems considered in this paper were that non-lactating rats with consequently frequent pregnancies grew faster than the rats which were allowed to lactate. They attained approximately the same ultimate weight. The virgins grew slower and were ultimately lighter than the other two groups. However, no attempt was made to ascertain whether the difference was due to the additional weight of the uterus and contents and mammary glands in the bred rats. Later (1930) he compared the growth of ovariectomized rats with virgins. He found that the spayed rats grew faster and became larger than the virgins, but they did not become as heavy as the bred rats. Measurements of body length taken at death indicated that the increased ultimate growth was not wholly due to fat deposition as the spayed rats were 172 mm. long as compared with 167 mm. for the virgins. These results confirmed those which had been obtained by Stotsenburg (1913), but agree only in part with those of Freudenberger and Billeter (1935).

Slonaker suggested that the greater growth of the spayed rats was due to the fact that they were less active and therefore were able to use more of their food intake for growth. Against this view is the conclusion by

[1] These studies were supported by a grant from the Rockefeller Foundation.

Donaldson (1932) that exercise increases the weight of rats. In view of the apparent contradiction it is probable that the factor is not exercise, but something else, probably an endocrine phenomenon.

While our work was in progress Cole and Hart (1938) compared the growth curves and ultimate weights of virgin, lactating, and parous non-lactating groups of rats. Their bred groups contained rats which were induced to breed prematurely at 28 to 31 days as well as those which were bred at the first estrus (50 to 88 days). They also had a group which was bred "normally" at from 50 to 80 days. They found that the first pregnancy accelerates growth and that the difference was increased with succeeding pregnancies. These rats were not carried through until they attained their maximum growth, although one group of 5 rats was carried almost to this point. Cole and Hart recognized the difficulty of comparing the growth curves of virgin and pregnant rats. They did not correct their growth curves for this factor and it is not clear when their weighings were made, though the curves are drawn at 40-day intervals. However, recognizing this factor, they compared the weights of carcasses without uteri and contents for pregnant or parturient rats and for virgins and still found a significant difference in weight. They also showed that the increase in growth during pregnancy is accompanied by an increased food consumption.

The problems of growth are so complex that no one experiment can be set up to settle every point which may be considered. It is also impossible to set up an experiment that will satisfy all the criteria which may be drawn up for the perfect experiment. The objectives have to be stated first and the conduct of the experiment designed to give the best answer within the framework of those objectives. Our objectives in this work differed from those of others who have worked in the field, so that our criticisms apply to their work only as it happens to fall within our own objectives.

Our objectives were: 1, to observe the effects upon the rate of growth and upon the ultimate weight of *a*, non-breeding; *b*, early breeding; *c*, late breeding; *d*, intensive breeding without lactation; *e*, ovariectomy, and *f*, "normal" breeding. By normal breeding we mean breeding at 100 days old, which allows of some further growth after puberty before the organism is subjected to the burden of reproduction and lactation. This compares with human custom and agricultural practice. 2. To set up our groups with sufficient numbers for adequate statistical analysis. 3. To determine the nature of the growth to some extent by x-ray studies.

MATERIAL AND PROCEDURE. Three hundred female rats at the normal weaning age (21 days) were divided into 6 groups of 50 each. The distribution of these young rats into each group was a random one except that litter mates were not put in the same group. Five female rats were kept

in a large colony cage, so there were 10 cages for each group. One male was placed in each cage in those groups in which the females were bred. The males were rotated weekly within each group so that a poor breeding male would not have much effect upon the breeding record of the females. When the males were a year old, but before the age of the male was a factor in determining the frequency of litters or young per litter, they were replaced by younger males.

The 6 groups were: 1. Bred late. When the females were 9 months old, males were placed with them. The young were reared to 3 weeks of age and the female was rebred.

2. Bred early. Males were placed with these females before the age of vaginal opening. These females were bred as young as possible. The young were reared to 3 weeks of age and the female was rebred.

3. Bred at normal age. When these females were 100 days old, males were placed with them. The young were reared to 3 weeks of age and the female was rebred.

4. Virgins. These females were never bred.

5. Bred at normal age—young killed. When these females were 100 days old, males were placed with them. The young were killed at birth and the females were rebred. Thus, these females were pregnant most of the time.

6. Castrates. These females were ovariectomized at about 40 days of age. At this age the vagina of only one female had opened.

The females were weighed at the beginning of the study and once every 28 days (irrespective of the reproductive state) throughout the experiment. The age and weight of the females at the time of vaginal opening were obtained. In those groups that were permitted to breed, weights of the females were obtained immediately after parturition, and also at weaning in those females that were permitted to suckle the young.

The females were observed twice weekly for pregnancy by palpation of the abdomen. Those females that were not permitted to breed were observed for any abnormality. Pregnant females were removed from the breeding cage to a small individual cage for delivery, and, for those females that were permitted to suckle, the rearing of the young. A complete reproduction record was kept, including the weights of the young, sexes, and weights of the females. Where young were allowed to suckle, the female and young were kept in the small round cage until the young were weaned (21 days of age), at which time the female was returned to the breeding cage. Where the female was not permitted to suckle her young, she was returned to the breeding cage as soon as the litter was recorded. This procedure was open to criticism in that there were generally fewer animals in the breeding cages (less crowding) in the groups that suckled their young. If a litter was born in the breeding cage (this occasionally

happened if pregnancy was not detected), the female and young were removed and recorded as soon as they were observed. A female that aborted was returned to the breeding cage immediately, but when a female lost her litter from failure to suckle, she was kept in the individual cage for the full 3 weeks.

All rats received the same stock diet (Maynard, 1930). They were kept in an air-conditioned room, maintained at an average temperature of 78 degrees F and a relative humidity of 50 per cent.

X-ray pictures of the right rear leg of representative animals from each group were taken at definite intervals in a study of bone growth. X-ray plates of the abdomens of several females that had parturition difficulties were also taken. Photographs of representative animals of each group and pictures of unusual abnormalities were taken as a routine procedure.

RESULTS. The results of the growth studies will be presented in three parts: I, age and weight of the females at the time of vaginal opening; II, growth curves; III, mature weight and the age when they reached the mature weight.

I. *Age and weight of the females at the time of vaginal opening.* The average age of the different groups of rats at vaginal opening (as was to be expected) was practically the same with the exception of the "castrates" (table 1). These ovariectomised rats were significantly older at the time of vaginal opening than any of the other groups. The average age varied from 58.6 to 61.1 days for all the groups except the castrate group which was 74.5 days.

The average weight of the females at the time of vaginal opening varied from 119.7 to 123.2 grams for all the groups except the "castrate" group which was 160.5 grams (table 1). From an analysis of variance it is evident that the heavier weight and older age of the "castrates" at vaginal opening is mathematically significant (odds 99:1, Fisher).

The coefficient of correlation for age and weight at the time of vaginal opening after the removal of the variance due to treatment, location of the animals, and interaction is +0.57. The regression of age on weight is 2.68 grams which means that for every day above the mean there is an average increase of 2.68 grams in weight. The regression of weight on age is 0.12 day. The necessary difference for significance for age is 2.45 days, and for weight 11.40 grams. Thus, when the weights of the groups are put on the same age basis (table 1) the difference between any two groups is not mathematically significant. On the other hand, the "castrate" opened at a significantly later age than any of the other groups (table 1).

It would appear that ovariectomy has brought about an increase in age at the time of vaginal opening and also permitted a more rapid growth rate. Ovariectomy, however, did not increase the weight of the animals

at the time of vaginal opening except by increasing the age. These data are in accord with results of Asdell and Crowell (1935) which indicated that age is more closely related to vaginal opening than weight.

The coefficient of variability was smaller (4.89 per cent) for the age than for weight (10.55 per cent) at the time of vaginal opening. Of the two, age seems a more important determining factor for the time of vaginal opening than weight. This also bears out the results of Asdell and Crowell (1935) in which it was found that rats retarded in growth by caloric deficiency opened at a later age than usual, but at a much lower weight than is customary. Engle, Crafts and Zeithaml (1937) have shown that skeletal size, as measured by body length, has a closer correlation than either age or weight with the time of vaginal opening. An examination of their data shows that the coefficient of variability for body length is smaller than that for the other factors mentioned. However, it must be

TABLE 1

Age and weight of rats at the time of vaginal opening

GROUP	UNCORRECTED AVERAGE AGE	AGE CORRECTED FOR WEIGHT	S.E.	UNCORRECTED AVERAGE WEIGHT	WEIGHT CORRECTED FOR AGE	S.E.
I. Bred late	59.7	60.5	±.88	121.2	127.4	±4.11
II. Bred early	59.4	60.4		119.7	127.5	
III. Bred normal	61.1	61.7		123.2	126.4	
IV. Virgins	60.8	61.7		120.8	124.8	
V. Young killed	58.6	59.5		120.7	130.6	
VI. Castrates	74.5	70.6		160.5	127.5	

TABLE 2

Percentage instantaneous growth rates of virgin and castrate rats

	VIRGINS	CASTRATES
1st month after weaning	50.05	48.27
2nd month	23.87	28.57
3rd month	8.86	11.92
4th month	2.49	6.07
5th month	2.96	1.69
6th month	1.41	1.93
7th month	1.75	1.25
8th month	0.76	1.80

remembered that the body length and weight (also age to a lesser extent) are closely related. Their data give a closer fit for age than for weight.

Since the "castrate" females were ovariectomized only a short time prior to the normal age of increased ovarian function, it is possible that sufficient hormonal influence to cause some effect had resulted prior to the operation. Some of the other glands have been found to contain estrogenic substances but it is not known whether they secrete those substances or act as temporary stores for the estrogens.

There would seem to be three possibilities: 1. That the effect produced by the ovaries prior to the operation was sufficient to cause vaginal opening at a later date than usual. 2. That some other gland stored or secreted substances similar in action to the estrus producing hormone of the ovary. 3. That the vagina might eventually open from non-hormonal influence.

Our groups are all similar in showing vaginal opening at the time of a

marked change in the slope of the growth curve (weight increment). The "castrate" females showed the same relationship as the unoperated rats between vaginal opening and a slower growth rate.

No abrupt change but a gradual slowing of the growth gradient may be observed in the graphs (fig. 1). The potential growing period of the rat is extremely lengthy since many of the females continued to grow after as many as 15 litters were produced. Rats that were retarded in early life by restricting the quantity of food also showed an ability to grow at

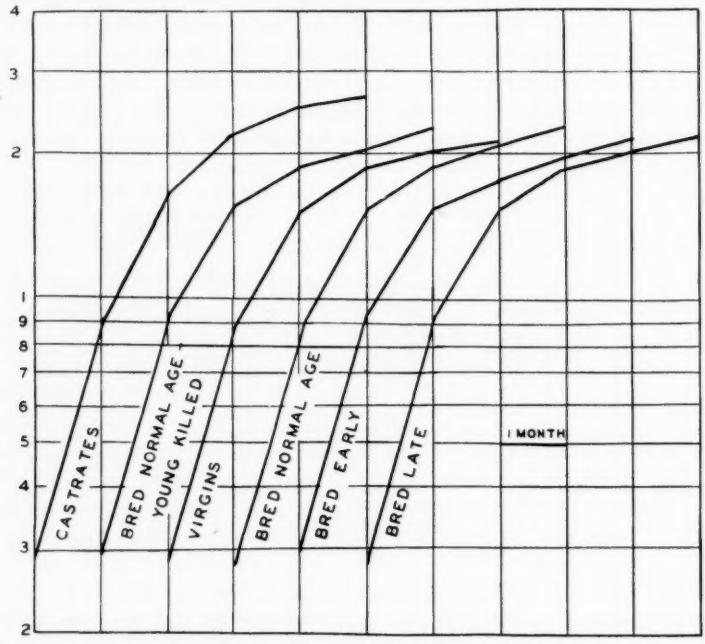

Fig. 1. Growth curves of female rats as influenced by reproductive condition The slopes of the curves on semi-log paper are shown in this figure.

a very old age (McCay et al., 1939). This may be related to the fact that the ossification of many of the epiphyses occurs very late, if at all, in the life of the rat.

Vaginal opening occurred when the "virgins" weighed 52.5 per cent and the "castrates" 54.6 per cent of their weight at the time of the plateau in growth. This rather small difference between the two groups indicates that the event in the two groups is related to growth but not to eventual weight (since this is a later event). It would be logical to conclude that vaginal opening is not a function of eventual weight but that the interplay

of forces which produces a given eventual weight also fixes vaginal opening at a definite epoch in this growth.

II. *Growth curves.* Growth, as measured by weight, is sometimes not an accurate indication of change because weight is influenced by other factors than skeletal size such as the degree of fatness. Our data on weight of the rats show such a close agreement with increase in length of the tibia (measured by x-ray plates) that we have considerable confidence in them. Undoubtedly some of the increase in weight resulted from more fat but the trends in the growth curves coincide very closely with the trends in the length of the tibia.

A difficulty in presenting growth curves is to decide in what form the data shall be used. In this experiment, the main difficulty is to decide what weight shall be used. If weights taken at definite intervals are used, there are no complications for the virgin and ovariectomized groups. But in the parous groups, the additional weights of uterus and contents, together with mammary glands must be taken into account. These are all more or less periodic variables not in unison from rat to rat. One may assume that their average value will be the same for each group. This is an unjustifiable assumption as the reproductive regime, which varied from group to group, may affect the increase due to these factors. This is especially true for the "bred at normal age, young killed" group in which the litters were larger and were born at more frequent intervals. A second possibility is to take non-pregnant weights (either following parturition or at the weaning of the young), and to average the weight for the average age at each litter. This has the advantage of eliminating the variability due to the uterine contents. It presents, however, a large age variability as the rate of breeding was not uniform, while the weights in the non-bred groups were taken at regular intervals. The main disadvantage, inherent also in the method first suggested for plotting, is that the weight of the uterus and of developed mammary glands is not taken into account. It was decided that this must be corrected. Accordingly 25 rats were bred at the normal age (100 days), allowed to suckle their litters and mated again at weaning. Immediately after their second parturition each was killed and a virgin control of the same age was killed at the same time. The uteri, ovaries and mammary glands of each rat were dissected out and weighed. The average weight of these organs in the virgins was subtracted from the corresponding average for the parous rats. The difference was 8.2 grams. For simplicity's sake the curves for the parous rats were dropped 8 grams at each of the points determined by parturitions. The resulting curves therefore represent a true record of the effect on growth of the reproductive factor uncomplicated by more or less periodic fluctuations due to the variable reproductive state within the group.

The weight of the parous animal minus the correction factor (8 grams) was also the weight used for statistical treatment in testing the significance of the differences in the growth curves.

At the beginning of the experiment (i.e., weaning) the groups were very uniform in weight. It was not until the rats were about 80 days of age that differences in weight were noticeable. At this age the "castrates" were significantly heavier and had longer bones than any of the other

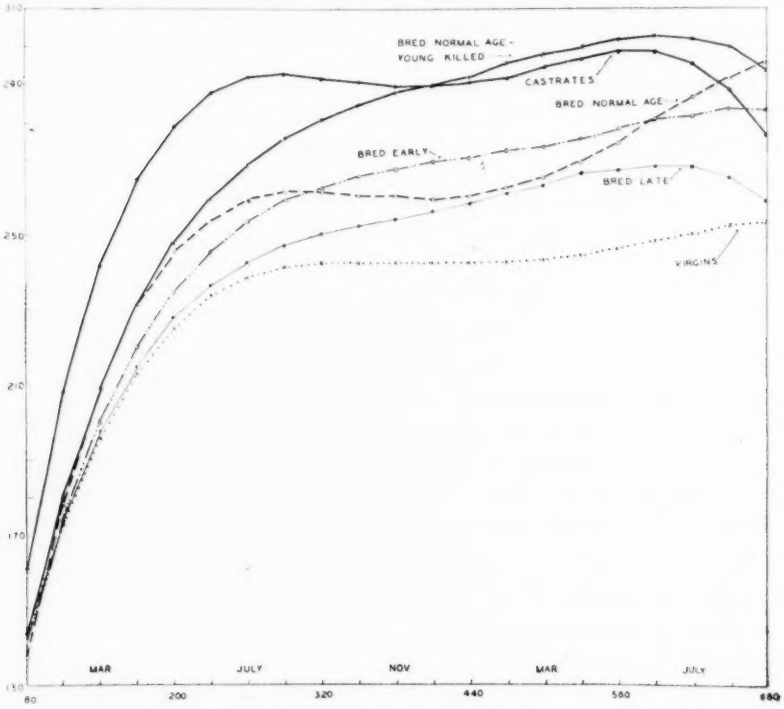

Fig. 2. The growth of female rats from 80 to 680 days of age as influenced by reproductive condition. These curves are drawn from figures smoothed by polynomials and are conventional growth increment curves.

groups (figs. 2 and 3). The slopes of the growth curves for the first half year of life are shown in figure 1. The differences between the groups from 80 to 680 days of age are shown in figure 2. Figure 1 is plotted on semi-log paper to show the changes in growth rate, while those in figure 2 are conventional growth increment curves to illustrate the weight differences between the groups. These curves have been smoothed by use of polynomials (Golden, 1939). The test of significance was applied to the smooth

curves by finding the necessary difference for each possible group comparison.

The "castrates" were heavier than any other group from 80 to 410 days of age (fig. 2), but the difference between them and the "young killed" group was not significant after 290 days of age. The latter group eventually surpassed the "castrates" in weight as did also the "bred normal" and "bred early" groups but none of these differences were significant. The reason for the eventual superiority of weight of the "bred early" and "bred normals" was the decline in weight of the "castrates" due to an earlier premortal weight loss. The "castrates" held a superior weight

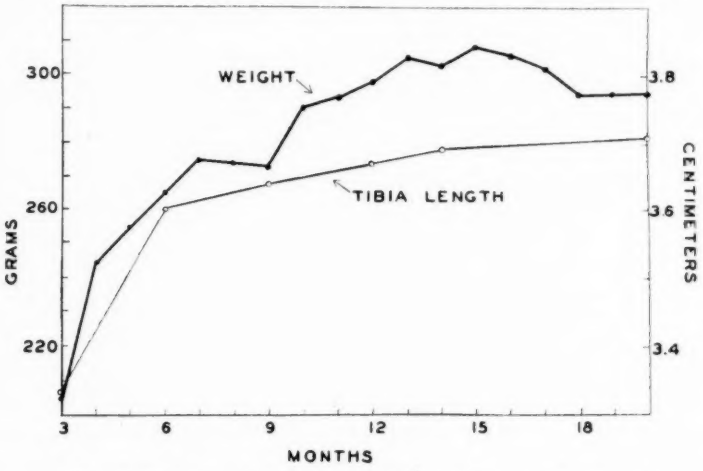

Fig. 3. Weight and tibia length increase of castrate female rats

position over the "virgins" throughout their lives. This was mathematically significant after 80 days of age. They were heavier than the "bred-lates" but the difference at 650 and 680 days of age was not significant mathematically since the variability was large at these times. The "castrates" were significantly heavier than the "bred normal" or "bred early" groups from 80 days of age to 560 for the "bred early" and 590 days for the "bred normal" group.

The "young killed" group was significantly heavier than the "virgin" and "bred late" groups from 140 days of age throughout their lives (fig. 2). They were significantly heavier than the "bred early" group from 170 days to 620 days of age. Even though the last 3 months were not mathematically significant, they maintained a superior weight throughout.

The "bred normal" group did not differ from the "bred early" group sufficiently to show a statistical significance. The two groups, "bred

normal" and "bred early," were significantly heavier than the "virgins" from 170 days of age throughout life.

The "bred late" group was not different from the "virgins" in weight (since they were really another "virgin" group) until they were bred. They made a spurt in growth following breeding and maintained a significantly heavier weight than the "virgins" from 380 days of age until premortal weight losses ensued, at 620 days of age. The "bred late" animals never attained the weight of the "bred early" or "bred normal" groups but were intermediate in weight between them and the "virgins." They were significantly lighter than the two former groups before breeding and at the end of the curve, but not while the effect of breeding made itself felt.

The "castrates" grew at a much more rapid rate in early life than any of the other groups but reached a plateau[2] at about 260 days of age, growing very slowly later in life. Thus, ovariectomy in the rat increased the rate of growth and tended to shorten the growing period. These females did not assume the old age adiposity which is often observed in castrate males.

The "bred early" and "bred normal" groups reached a plateau at a slightly greater age than the "castrates." Afterwards they continued growing at a relatively more rapid rate.

The "bred late" group followed exactly the same growth curve as the "virgins" until they were bred, then grew rapidly for a time and reached a plateau only shortly before the premortal weight losses began.

The "virgins" reached their plateau at about the same age as the "castrates" but had grown so slowly prior to this time that they were much smaller.

The "young killed" group grew at a relatively rapid rate for a longer period of time than any of the other groups but their rate of growth was not equal to that of the "castrates" earlier in life.

It would seem that breeding not only increases the rate of growth but also extends the growing period. Ovariectomy only increases the rate of growth but does not increase the growing period.

A comparison of the percentage instantaneous growth rate for the first 8 months after weaning, between the "virgins" and "castrates" is given in table 2.

It will be seen that, almost throughout, the rate of growth of the "castrates" is well above that of the "virgins." In order to show the agreement in growth trend and increase in tibia length, the curves for tibia length and growth for the 10 rats x-rayed in the "castrate" group are

[2] The term "plateau" is relative, and indicates that at this time the rate of change is very slow.

presented (fig. 3). Also, curves for the increase in length of tibia for all the groups are shown in figure 4.

It is interesting to compare the groups according to bone length (fig. 4). The "castrates" excelled in bone length in the early part of life but were later overtaken and far surpassed by the "young killed" group. The "virgins" and "bred late" females showed very close agreement in bone length until the latter group was bred. At this time the bones of the "bred lates" showed a marked increase in growth, but they never reached the "bred normal," "young killed" or "castrate" sizes. The "virgins'

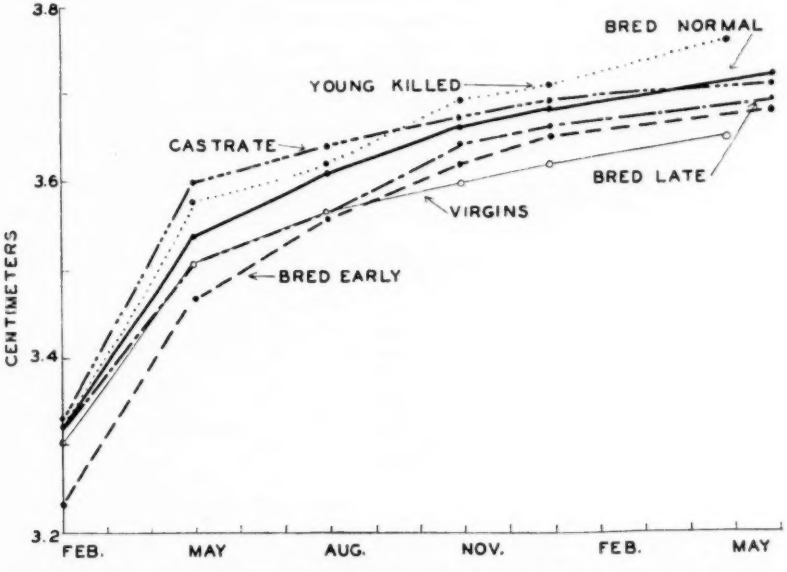

Fig. 4. The length of tibia of 10 representative animals from each group shows the growth in skeleton as influenced by reproduction.

had the shortest bones of all at maturity. The "bred early" group apparently do not fit into the picture as clearly as the others. Either the rats randomly selected for x-ray studies were smaller than the average at the beginning or the early lactation was a set-back to bone growth. Bone calcium studies reported elsewhere (Chen, 1939) indicate that the second view is the correct one. Also a study of the weights for the rats used in the x-ray studies shows that the first explanation is untenable, because these rats did not differ greatly from the average for the group. The bones of the rats in this group grew more rapidly later in life than those of rats in the other groups so that the set-back was not permanent.

III. *Mature weight and age.* The mature weight of the female rats was determined by taking the heaviest weight. In those animals that were breeding, this heaviest weight was based on weight at parturition and the weight was corrected to that of the "virgins" by subtracting the difference in mammary gland and uterine weights previously used. The mature age was the age of the animal at the time of the heaviest weight.

"Analysis of variance" was calculated for the mature weight and age and correlations between age and weight were made within each group.

The "between group" variance was significantly greater than the "within group" variance (error), (odds of 19:1), therefore we conclude that the difference between some of the groups is of significance.

The "young killed" and "castrate" groups are significantly heavier than the "virgins" at mature weight but no other group comparison shows a mathematical significance. From a biological standpoint, we should attach some significance to the other figures because the other groups show

TABLE 3

Mature weight and age of rats by groups

	MATURE WEIGHT	MATURE AGE
	grams	*days*
I. Bred late	288.3	492.5
II. Bred early	302.3	500.8
III. Bred normal	300.6	477.8
IV. Virgins	263.9	461.2
V. Bred normal age, young killed	315.9	497.1
VI. Castrates	317.3	445.0

The necessary difference for significance for weight is 44.5 grams, and for age is 55.6 days.

TABLE 4

Relative mature weights of rats (virgins, 1.0)

I. Bred late	1.09
II. Bred early	1.15
III. Bred normal	1.14
IV. Virgins	1.00
V. Bred normal age, young killed	1.20
VI. Castrates	1.21

a mature weight more or less in proportion to the amount of reproduction they had undergone when they were compared with the "young killed" females.

The mature age was quite variable and the differences between the groups failed to show a mathematical significance (odds of 6:1 for parous vs. non-parous rats) but some interesting relationships were found. The mature age was lowest for the "castrates," which again indicates that ovariectomy increased the rate of growth but not the growing period. The "virgins" showed the next lowest mature age which means that they did not grow for a very long period. All the bred groups grew for a longer time than the non-bred groups and at a more rapid rate than the "virgins." Although the growth rate earlier in life was low, the "bred late" group grew for a long period and more rapidly than the "virgins" later in life; therefore, they were intermediate in mature weight between the "virgins" and the other bred groups. They experienced the pregnancy stimulus later in life and to a smaller degree than usual.

When the mature weight is expressed relative to the "virgins" the figures shown in table 4 are obtained.

All our females showed a loss of weight in old age. Since the "castrates" reached the mature weight on an average earlier than the "young killed" group, the influence of premortal weight loss was greater for the "castrates."

The age at which old age weight losses first occurred together with the corresponding weight has been determined. Since our animals did not all complete the normal life span,[3] figures for the age and weight at death cannot be obtained. The only figures possible are the weights with corresponding ages for the last weight prior to death. These data are presented in table 5.

TABLE 5

Weight loss from old age in the rat

GROUP	AGE WHEN WEIGHT LOSS BEGAN	WEIGHT WHEN WEIGHT LOSS BEGAN	AGE AT DEATH*	WEIGHT AT DEATH*	WEIGHT LOSS
	days	*grams*	*days*	*grams*	*grams*
I. Bred late	525	281.8	645	228.3	52.5
II. Bred early	537	291.8	618	256.0	35.8
III. Bred normal	534	287.7	603	254.8	32.9
IV. Virgins	549	257.7	633	227.4	30.3
V. Bred normal age young killed	543	306.1	612	262.0	48.1
VI. Castrates	486	317.2	648	253.3	63.9

* About half of these rats were killed by heat; therefore, these ages and weights are not normal life span figures.

TABLE 6

Correlation coefficient between mature weight and age

I. Bred late	+0.588*
II. Bred early	+0.095
III. Bred normal	+0.150
IV. Virgins	+0.505*
V. Bred at normal age, young killed	+0.623*
VI. Castrates	+0.309

* These are statistically significant.

It may be seen from this table that the "castrates" began to lose weight at a younger age than any of the other groups and lost more weight than any of the others.

The correlation coefficient between mature weight and age for each groups follows (table 6).

The meaning of these results is obscure. One might assume that a positive correlation indicated that our animals reached the heavy weight because of a longer growing period, whereas a failure to show a significant correlation would indicate that the animals reached the large weight because of a more rapid growth rate. On the whole the correlation became

[3] The air conditioning plant failed to function one night and approximately half of the animals died from excessive heat and humidity. This occurred when the rats averaged 667 days of age. As the rats were all born within a very few days the accident did not affect the data included in this paper. For this paper the experiment was ended at 667 days.

less the earlier the breeding, yet that does not appear to be the paramount consideration since the "kill young" group showed the closest correlation of all. Probably lactation is a disturbing factor (absent in the "kill young" group) which upsets the normal relationship. There is some support for this view in the fact that the "bred late" group, with a high correlation, had more difficulty in lactating so that they did not experience the drain on their systems that affected the other groups.

Discussion. Estrogens in large amounts have been shown to have a retarding effect upon growth in the male and female rat (Bugbee and Simond, 1926; Spencer, Gustavson and D'Amour, 1931; and Freudenberger and Clausen, 1927). Whether this retarding effect is due to the inhibiting effect of estrogens upon the pituitary, causing the latter to secrete less growth hormone, or direct effect upon growth is not known. The injection of growth hormone into normal females (Cole and Hart, 1938) has given good growth. What would the result have been if estrone had accompanied the injections of the growth hormone?

Ovariectomy eliminates the estrogen supply and a more rapid rate of growth results because there is no inhibition of growth due to this factor. Some evidence points to the direct retarding effect of estrogens. Estrogens in large quantities cause an endosteal bone formation (Gardner and Pfeiffer, 1937) and might cause the bone growth center completely to ossify so that an increase in length of long bones would be impossible.

This inhibition of growth by estrogens appears to be a positive effect since the normal male reaches a greater weight than the female. Castration of the male does not alter the growth rate or mature weight according to Stotsenburg (1909), though Slonaker (1930) records a stimulation as a result of the operation. This is less, however, than the similar stimulation after ovariectomy. The inhibition must be classed as a secondary sexual character, with a behavior similar to that of feather shape and coloration in a breed such as the brown leghorn fowl. The neuter female approaches the male in this respect.

Pregnancy causes an increase in growth rate in rats (Cole and Hart, 1938; Slonaker, 1927). Eckles (1916) has shown that cows are more efficient while pregnant than when open. It would appear that storage accompanies pregnancy and that the lactation drain removes at least part of the storage. Does the corpus luteum influence the physiology of the animal either directly or through the pituitary so that the body will take on these stores? Brody et al. (1938) have shown that the metabolism of the pregnant animal is no greater than is expected from the weight increase so there is no excess energy expenditure due to pregnancy. Wang (1925) has shown that food intake is greater during pregnancy, while he and also Slonaker (1924–5) have shown that less exercise is taken during pregnancy. It might be that during pregnancy little estrogen is secreted

and that because of a greater food consumption and less energy waste by activity, the pregnant female is able to store large supplies or to grow. The corpus luteum may influence the pituitary by causing an opposite effect to that of estrogen. It may thus bring about growth. However, there is considerable evidence for many species other than the rat that estrogens are particularly high during pregnancy. Does the corpus luteum hold this estrogen effect in check?

That pregnancy is a definite stimulus to growth is shown by the "bred late" group which had practically plateaued before breeding and which made a sudden spurt in growth when the rats were bred. (Does it remove an inhibitor sufficiently strong to overcome the growth urge which has now become weak?) Also the continued growth of the "young killed" group is indicative of a stimulating effect of pregnancy, because there was no drain of lactation in this group and the continued stimulation for growth resulting from pregnancy was sufficient to cause a marked increase in the size of the females. It might be that pregnancy removes the inhibition for growth (estrogens). In the "bred late" group a smaller response than usual was possible because the growth urge had become weak.

The stimulus to growth during pregnancy is stronger than the drain during lactation in the rat because the groups that suckled young far surpassed the "virgin" group in growth. It would be tempting to compare our results with similar results obtained from other species with heavy lactation demands. But the pregnancy stimulus has not been observed either in the sow (McKenzie, 1928) or in the cow (Eckles and Swett, 1918). One would not expect the stimulus to growth from pregnancy in a dairy animal if she were permitted to lactate heavily while pregnant.

There is abundant evidence that the age of the female at puberty (vaginal opening or first conception) is influenced by nutrition. It also varies with strains. Ovariectomized females experience vaginal opening in the absence of ovaries (and presumably in the absence of estrogens). This indicates that factors other than ovarian hormones play a part in this phenomenon. Vaginal opening occurs at a rather definite period in growth, when the growth curve becomes markedly less steep. This indicates that factors influencing growth also influence vaginal opening. The opening of the vagina does not seem to be related to adult size. It is part of the maturation process which is hastened by the presence of the ovaries.

Continued pregnancies increase the growth rate and also prolong the growing period whereas ovariectomy increases the growth rate only.

All the work upon the effect of breeding upon growth is in agreement (Cole and Hart, 1938; Slonaker, 1927) in showing that pregnancy is a definite stimulus to growth even though different methods were used. Slonaker grouped his rats according to reproductive performance and Cole and Hart obtained pregnancy in their rats at a much earlier age than

normal. The data reported by us are especially striking as it is shown that even relatively old rats will grow under the stimulus of pregnancy.

We speak of the pregnancy stimulus but we have pointed out that it is probably a removal of the inhibitor in the normal females. It is also a question whether pregnancy in itself or the corpus luteum is the effective factor. The balance of evidence is in favor of the second view as Slonaker (1929) has shown that during pseudopregnancy a similar stimulus to growth is found. We conclude therefore that the corpus luteum is the probable effective agent. During ovariectomy and pregnancy cellular changes in the pituitary have been described by many workers. These changes may have a bearing on the problem.

As our results are so consistent one is tempted to speculate upon the practical application of these studies. It is clearly indicated from the data that early breeding in a species in which milk production is not a highly specialized phenomenon does not decrease the final adult size. If size were desired, as in show animals, breeding without lactation might be desirable.

In a species like swine, one can see no economic benefit from deferring breeding if the female is well grown or from keeping the sow open for rest periods, except that a rest after the considerable lactation drain might be advisable. The effects of different rest periods in the rat are now under investigation.

SUMMARY

Data are presented on the age and weight of female rats at the time of vaginal opening which demonstrate that the vagina opens in the absence of the ovaries but at a greater weight and age. It is suggested that the factors influencing growth are also concerned with vaginal opening.

Ovariectomized females grow more rapidly early in life than virgins or breeding animals, but the growing period is no longer than for the virgins.

Animals breeding but not lactating grow more rapidly than lactating animals or virgins and grow for a longer time than virgins.

Breeding, lactating animals grow more rapidly than virgins and for a longer time but do not approach the growth rate of ovariectomized or breeding-non-lactating females. They grow for a longer time than the ovariectomized rats.

Virgins grow at a slower rate and reach a plateau in growth earlier than breeding animals. They grow at a slower rate than ovariectomized rats but these two groups reach their plateau at the same age.

Early bred animals may have a set-back from the drain of lactation early in life, but they reach the same adult size as animals bred later.

Even after the rat has reached the growth plateau, breeding stimulates growth. Late bred animals never attain adult size equal to other breeding

animals but they surpass the virgins. They experience the pregnancy stimulus late in life and respond, though not to the extent that they could have done earlier. The residual growth influence is probably less at this time.

It is suggested that estrogens inhibit growth possibly through an inhibiting action upon the pituitary and that the corpus luteum removes this inhibition. Ovariectomy has a similar effect by direct removal.

Ovariectomized rats experience the pre-mortal loss of weight at an earlier age than other rats.

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THE EFFECTS OF ADRENALINE AND OF NERVE STIMULATION ON THE MECHANICAL AND ELECTRIC RESPONSES OF UTERINE MUSCLE[1]

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It has recently been suggested (Bozler, 1938a, b) that smooth muscle behaves like a single unit. Electrical stimulation of the uterus during estrus was found to give rise to responses of an apparently all-or-none character which traveled throughout the muscle. In addition, large demarcation potentials were observed. Furthermore, it can safely be assumed that the large spontaneous contractions of such muscles are conducted. The uterus thus presents a means of determining whether adrenaline or nerve impulses produce their effects by setting up all-or-none propagated disturbances. In the present study an attempt to answer that question was made by separately recording the activity of the two ends of a single uterine horn in response to the stimulation of one of them.

METHOD. The uteri of non-pregnant cats and rabbits were used. Estrin (usually 20,000 R. U., Progynon B) in oil was injected intramuscularly three to eight days before the experiments. In the rabbits oöphorectomy was ordinarily performed prior to the injections in order to avoid the possibility of pseudopregnancy.

On the day of the experiments the animals were stunned by a blow on the head or anesthetized with dial, and the uterus removed for *in vitro* recording or arranged *in situ*. In the former case, a trough was prepared with a transverse partition in the center and immersed in a water bath held between 36° and 38°C. The uterus was slit along one side and fixed horizontally with pins thrust through the center into the partition of the trough. Either end of the uterus could thus be exposed to different solutions. To prevent cooling and drying of the center a bottle was arranged to supply drops of warm Ringer's solution (as modified by Van Dyke and Hastings, 1928) to the exposed portion. Recording was effected by threads led over pulleys to light isotonic levers equipped with frontal writing points. For *in situ* recording, the rabbit uterus was care-

[1] A preliminary report of these experiments was published in the Proceedings of the American Physiological Society, 1938, p. 150.

fully freed from as many mesometrial attachments as possible without damaging the blood supply. In this species the mesometrial artery on each side divides at some distance from the uterus and sends separate branches to each end (cf. Reynolds and Kaminester, 1935). By fixing the uterus with a needle between the main arborizations of these arteries and applying electrodes to the periarterial plexuses, it was usually possible to obtain discrete stimulation of either the vaginal or the ovarian end. To prevent cooling and drying in some experiments the whole posterior portion of the animal was immersed in a constant temperature bath of Ringer's solution. In other experiments, especially if electrical recording was contemplated, the room temperature was raised to about 34°C. and a high humidity was maintained by boiling water; the uterus could then be suspended in air either *in situ* or removed from the animal.

The electrodes used to lead off the action potentials were silver-silver chloride half cells connected to the animal through Ringer-agar wicks. One pair of electrodes was applied to each end of the uterus and led to a selector switch so that rapid shifts could be made from one to the other. The responses were amplified by means of a five-stage condenser coupled amplifier and recorded from a DuBois oscillograph. In a few experiments records of muscular contraction were made with a torsion wire mirror myograph simultaneously with the electrograms; ordinarily mechanograms were obtained on a smoked drum in the usual manner, the times at which electrical records were obtained being signalled on the drum by a signal magnet and key.

Results. *A. General observations.* As is customary in uteri during estrus, the preparations showed large spontaneous contractions of slow frequency. No procedure was attempted to eliminate this activity because it was deemed that such elimination could not be obtained without producing unphysiological conditions. The recording method sharply separated the contractions of the two ends of the horn. In most preparations one end, usually the ovarian, was seen to contract before the other. This confirmed the supposition (based on its faster rhythm when recorded separately) that the ovarian end is the usual pacemaker of the estrous uterus (Wislocki and Guttmacher, 1924).

The spontaneous activity complicated the evaluation of the effects of electrical stimuli applied to the muscle. Responses to single d.c. pulses, however, were clearly recognizable. Although it was sometimes possible to sum the effects of a few stimuli when applied at slow frequencies (Bozler, loc. cit.), large contractions of long duration, such as were always produced by adrenaline in the rabbit uterus, were never seen. High frequency stimulation did, however, elicit such contractions, an effect undoubtedly due to nerve stimulation (see section D).

The muscles used were often very susceptible to mechanical stimuli,

large conducted contractions frequently being caused by stretching or pinching, especially when these stimuli were applied toward the end of the relaxation period. In view, however, of the difficulty of evaluating the effects of mechanical or electrical stimulation, especially in uteri of rapid frequency, the presence of large conducted spontaneous waves was ordinarily taken as sufficient evidence of estrus and therefore of electrical excitability.

B. Effect of adrenaline on the non-pregnant cat uterus (n.p.c.u.). Small doses of adrenaline added to the solution bathing one end of a uterine horn decreased the amplitude of the spontaneous contractions of that end only

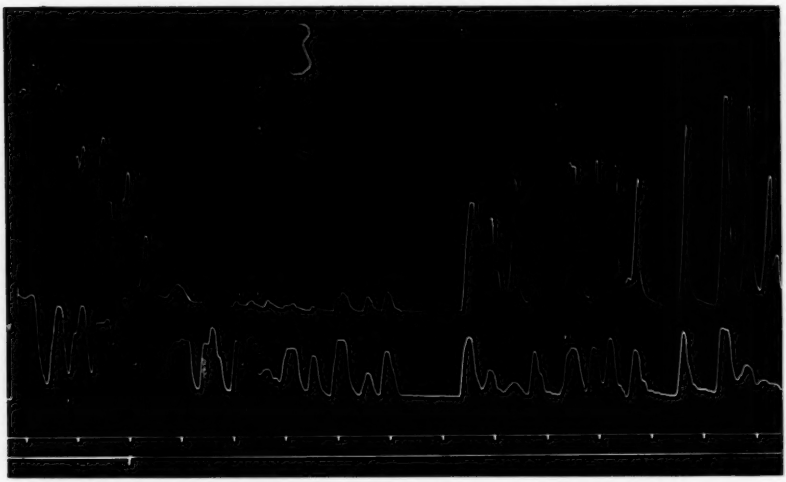

Fig. 1. Estrus cat uterus in vitro. Upper tracing, vaginal end; lower, ovarian. At signal adrenaline 10γ added to solution bathing vaginal end. In this and the succeeding figures the time signal indicates 30 sec. intervals.

(figs. 1 and 2). If this end was the one at which the beats originated, slowing of *both* ends was produced (fig. 2). The amplitude of the following end either remained the same, or, owing apparently to the longer rest period, actually increased (fig. 2). No change in rate ordinarily followed adrenaline treatment of the end opposite to the pacemaker, although its amplitude was always seriously reduced (fig. 1). In a few cases in which large doses were employed, dissociation of the rhythm appeared, the treated side becoming the slower one.

Changes in the base line of either end were seen rarely, and then only when there was slowing of a rate which was originally so high as to prevent complete relaxation between beats (cf. the lower tracing in fig. 2).

C. Effects of adrenaline on the non-pregnant uterus of the rabbit. When adrenaline was added to the fluid bathing one end of the uterus, it contracted strongly for a period of several minutes. The height of this contraction was variable in relation to the height of the spontaneous activity. In preparations which exhibited very large well-coördinated contractions of slow frequency, adrenaline responses were frequently no higher than the maximum spontaneous contraction, but only greatly increased in duration. Superimposed on these contractions, especially during the relaxation period, were smaller contractions of more than the normal frequency (figs. 3 and 5). The untreated side usually showed an

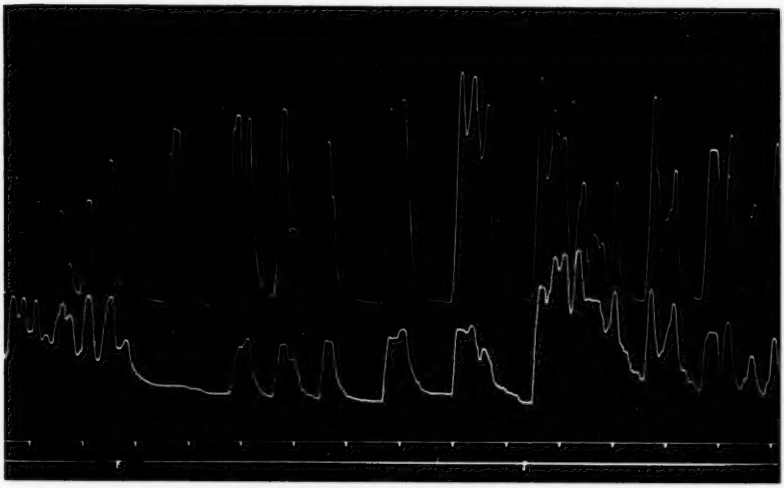

Fig. 2. Same as figure 1. First signal, adrenaline 10γ added to solution bathing ovarian end. Second signal, wash out.

increase in frequency with a decrease in amplitude (figs. 3 and 5) (the converse of the situation in the n.p.e.u. treated with adrenaline, section B). Sometimes the increased frequency of the untreated side was not very marked, consisting of occasional interpolated beats; on other occasions, the rhythm broke up almost completely into a series of rapid small contractions. Figure 3 illustrates an intermediate instance. Close inspection of the uterus or the recording levers showed that the end in contact with adrenaline always took over the function of pacemaker irrespective of which end previously set the rate.

Exceptionally the tendency of the untreated end to follow the frequency set up by the other resulted in a slight summation of contractions. In only one case, however, was this very marked. While the ovarian end of

an unusually active *in situ* preparation was recovering from previous adrenaline stimulation, adrenaline was applied to the vaginal end. The usual frequency increase occurred in both, accompanied by a prolonged contraction, not only of the vaginal end but of the untreated ovarian end as well. It seems likely that the long, fused contraction represents the unusual ability of a very active muscle to follow the frequency of the treated end (see discussion). Conversely in uteri of very low activity, i.e., pseudopregnant preparations, which showed few spontaneous contractions, adrenaline caused a long, smooth response of the treated end with no effect on the untreated portion. After the activity of both ends of such preparations had been increased by a moderate application of adrenaline

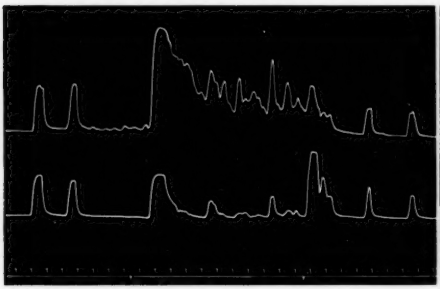

Fig. 3

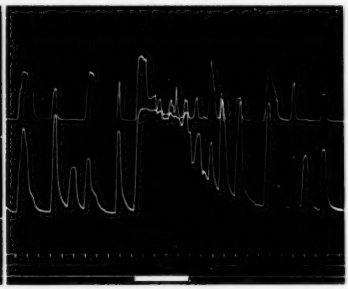

Fig. 4

Fig. 3. Estrus rabbit uterus in vitro. Upper tracing ovarian end; lower, vaginal. First signal adrenaline 100γ added to solution bathing ovarian end. Second signal, wash out.

Fig. 4. Estrus rabbit uterus in situ. Electrodes applied to periarterial plexus supplying vaginal end. Upper tracing, ovarian end; lower, vaginal. At signal, stimulation by inductorium, coil distance 6 cm.

to the entire muscle, typical results, as in figure 3, could be produced by further application of adrenaline to one end.

D. Effects of localized nerve stimulation. Stimulation of the nerve fibers distributed to one end of a single uterine horn produced results paralleling those of adrenaline application. Two methods were employed: stimulation of the plexus surrounding the artery to a particular end, and application directly to the muscle of induction shocks too short to activate the muscle itself, i.e., shocks which were individually ineffective. The results of the two methods were entirely comparable.

Localized effects of nerve stimulation were obtained in every experiment, but the use of very high intensities, especially in the region of the mesometrial attachment, sometimes led to contractions of the supposedly unstimulated end, a result doubtless attributable to spread of current.

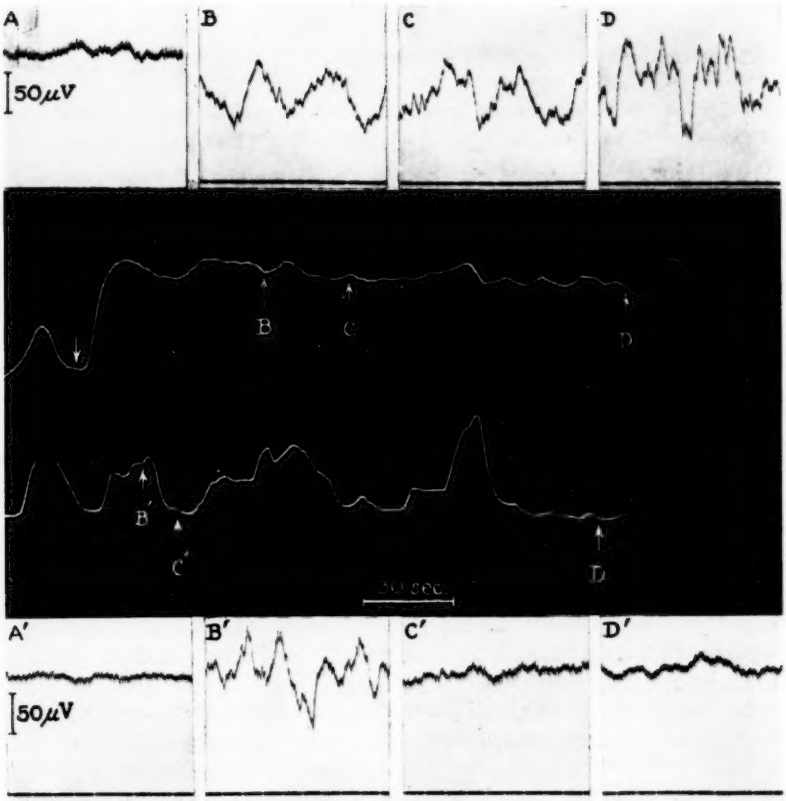

Fig. 5. Estrus rabbit uterus in situ. Upper kymograph tracing, ovarian end: lower, vaginal. Correlation of electrical with mechanical activity. The upper and lower rows of electrograms were obtained from the ovarian and vaginal ends respectively as indicated by letters and arrows. *A* and *A'* are records of the base line taken during quiescent periods shortly before the beginning of the kymograph record. Time signal of photographic records, 0.2 sec. At ↓ two drops of adrenaline 1:100,000 were applied to the ovarian end directly.

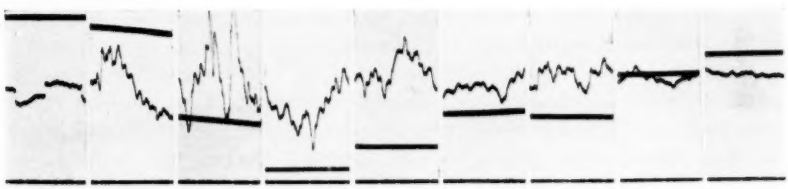

Fig. 6. Simultaneous record of mechanical and electrical responses of the estrus rabbit uterus separated from the animal and suspended in warm moist air. Isometric torsion wire myograph. First record, base line and calibration, 20μ V. Second record, immediately after adrenaline 1:10,000 applied to muscle directly. Subsequent records at 6 sec., 40 sec., 70 sec., 4 min., 6 min., 10 min. and 12 min. later.

A typical instance of a localized fused contraction accompanied by a marked increase in frequency, but no sustained contraction of the unstimulated end, is illustrated in figure 4.

E. Correlation of mechanical with electric responses. The possibility that the prolonged contraction produced by adrenaline was a contracture unaccompanied by action currents arose as an explanation of its failure to be propagated to other parts of the muscle. The records of Eccles and Magladery (1937) suggest that action currents are absent during the later stages of contraction of the nictitating membrane induced by adrenaline, but no comparable data are available for the uterus.

Simultaneous records of mechanical and electrical activity during very long responses (10 min. or more) showed that electrical activity was present until virtual completion of the slow relaxation period. Very marked at first, the potentials tended to decrease as contraction continued. Bursts of increased electrical activity coincided with increases of tension superimposed on the relaxation phase (figs. 5 and 6).

Simultaneous records from both ends of a uterine horn showed that the part exposed to adrenaline went into continuous electrical and mechanical activity for long periods of time, while the untreated end exhibited sharply separated bursts of potentials correlated with the intermittent spontaneous contractions (fig. 5).

Discussion. These experiments, though tending to support the idea that the uterus may on occasion behave as a syncytium, point to sharp limitation of the concept. Under proper conditions spontaneous rhythmic contractions and the responses to electrical or mechanical stimulation are conducted throughout the muscle. On the other hand, changes brought about by adrenaline or by nerve stimulation, i.e., increases or decreases in rate or amplitude of spontaneous activity, and prolonged contractions, are apparently confined to that part of the muscle to which the stimuli are delivered. Increases in the rate of the untreated end of a rabbit uterus when adrenaline is applied to the other end are not to be taken as evidence of conduction of some hypothetical acceleratory effect of adrenaline. Such changes merely reflect the fact that adrenaline has raised the rate of the conducted spontaneous beats at their origin.

It must be emphasized here that the treated portion of the muscle is almost always capable of sustaining a higher rate than the untreated portion. Adrenaline therefore seems to enable the muscle with which it is in contact to follow a frequency higher than that of which the normal tissue is capable, a property which seems to fit with the shortening of refractoriness reported by Bozler.

The prolonged contractions of the rabbit uterus produced by adrenaline may also be reasonably referred to a local diminution of refractoriness which results in a frequency of rhythm so fast, or a prolongation of each

beat so great, as to allow a more or less complete summation of contractions (figs. 3 and 5). This would explain the failure of such contractions to involve untreated parts of the muscle, unless the responsiveness of those parts was unusually high as in the exceptional case described in section C, and would relate the contraction producing property of adrenaline very closely to its chronotropic effects. The presence of action currents similar to those present during spontaneous contraction (figs. 5 and 6) supports this view and argues against the supposition that these prolonged increases in tension are contractures. On the other hand, the absence of electrical activity in untreated portions of the muscle is in accord with the concept (cf. Cannon and Rosenblueth, 1937) that adrenaline does not, in such cases at least, set up disturbances which are conducted throughout the muscle. Furthermore, adrenaline in very active estrous uteri rarely produces appreciably more shortening than is found during the spontaneous contractions, although the duration is greatly prolonged. Though not conclusive, this fact suggests that adrenaline acts principally to increase the duration of contraction by increasing the frequency to the point of fusion. Increments of tension beyond that of the spontaneous beats would be brought about only when the spontaneous beats were not previously recruiting all elements simultaneously. In muscles showing very slight spontaneous activity the shortening produced by adrenaline would be proportionately more striking. Whether the same explanation would apply to such normally quiescent muscles as the nictitating membrane as a limit must remain for future work to decide.

A similar sort of explanation applies to the inhibitory effects of adrenaline. Slowing of the n.p.c.u. is only brought about by adrenaline when applied to the pacemaking end. If the other end is not capable of maintaining the high original rate itself, it must of course be slowed also, but this does not imply that the slowing activity of adrenaline is conducted in a direct sense. In fact, in many preparations in which the level of spontaneous activity was high throughout the muscle, as indicated by the tendency for the normal beat to originate in various locations, no detectable slowing of the distant segment was produced by adrenaline applied to either end. In such cases, untreated portions of the muscle were free to maintain a practically normal rate of local origin.

Decreases in the amplitude of the spontaneous beats may also be due to an increase in the duration of refractoriness or the hastening of its appearance in the separate muscle elements. Either factor or both working together would tend to decrease the number of elements simultaneously active in any given beat and so reduce its size. Lowering of the base line or relaxations such as are strikingly produced by adrenaline in the anestrous uterus were scarcely perceptible in most of the estrous preparations. Since these well-coördinated muscles may be thought of as being alternately

active or refractory throughout their extent, an agent which acted to prolong refractoriness would not be expected to increase the length reached by the muscle during its refractory phase if the rhythm is relatively slow. With faster rates or if coördination is poor so that all elements are never simultaneously inactive, slowing of the rate would lead to lowering of the base line. Such an explanation would account for the slight relaxation shown in the lower tracing of figure 2, and the more marked effects so well known in less well-coördinated muscles.

Thus it seems possible to explain all the phenomena encountered in this study on the assumption that a fundamental property of adrenaline is to modify the refractoriness of smooth muscle. In those organs which it excites, it may be looked upon as delaying the development of the refractory state which seems to occur during each spontaneous beat and decreasing its duration once it appears. Conversely in organs which it inhibits, it would hasten its arrival and delay its disappearance when once established.

The experiments involving stimulation of the nerve supply to a limited portion of the uterus emphasize the well-known similarity of nervous to chemical stimulation of smooth muscle (cf. Cannon and Rosenblueth, loc. cit.) and its dissimilarity to direct electrical stimulation. Single electrical impulses may under proper conditions give rise to conducted impulses which travel throughout the muscle, but they have never been shown to be capable of summation into fused tetani of long duration (i.e., as long as those produced by adrenaline) either locally or at a distance from the electrodes. No response to single shocks applied to the nerves has ever been described, but higher frequency stimulation does produce fused tetani of long duration (see C and D) strictly localized to the part stimulated, as does the application of adrenaline. Rate changes induced by the two agents are also identical. Incidentally the localization of the responses to nervous stimulation (fig. 4) casts doubt upon the notion of a continuous nerve net in the uterine wall unless conduction within it is assumed to be sharply decremental.

SUMMARY

1. The mechanical and electrical responses of the two ends of a single uterine horn in estrus were recorded in various conditions.

2. The spontaneous contractions, as well as responses to mechanical or electrical stimulation, were conducted throughout the muscle.

3. Responses to adrenaline or nerve stimulation were localized to the region exposed to those agents (figs. 1–5).

4. Electrical responses were observed to accompany contraction even when adrenaline produced very prolonged mechanical activity (figs. 5 and 6).

5. The observations of conducted activity are shown to be compatible with the idea that the uterus may behave like a single unit.

6. Adrenaline and nerve stimulation, on the other hand, do not set up disturbances propagated throughout the muscle, but find an explanation in the assumption that adrenaline and sympathin modify locally the refractoriness of the regions in which they act.

7. The similarity of nervous to chemical stimulation and the differences of nervous from electrical activation are again emphasized.

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THE DIAMETER OF RED BLOOD CELLS IN HEALTHY YOUNG WOMEN[1, 2]

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For diagnostic purposes increasing emphasis is being placed on the diameter of red blood cells. Despite this fact, very little information is available as to the mean diameter and dispersion of the red cell population of normal individuals. The information that is available is based largely on studies of adult men and is very limited for women and children. Furthermore, there are wide differences in the values reported for adults, attributable in part, no doubt, to the methods used in making the determinations.

The present study was undertaken with the view of augmenting the information available on young adult women. The results secured in this laboratory, together with such data as the authors were able to locate in the recent literature, are given in table 1.

PROCEDURE. The subjects used for the present investigation were 80 healthy college women ranging in age from 17 to 24 years, with 84 per cent falling between 18 and 20 years. To insure uniformity, all blood samples were taken between 8:00 and 9:00 a.m., with the subjects in basal condition. Repeat observations were made on some subjects, resulting in the analysis of a total of 250 blood samples. In addition, 27 daily observations, extending over a period of 32 days, were made on one subject.

The method employed was as follows. Finger-tip samples were taken, diluted with Hayem's solution, counting chamber charged, and one diameter of 200 round cells measured immediately. The time required for making these measurements at no time exceeded 120 minutes, and in most instances was less than 90 minutes. Since only one diameter was measured, it was necessary to select only round cells, thus introducing a certain degree of selectivity into these results. It would seem, however, that any error incurred would work in both directions and tend to neutralize

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[2] Approved for publication by the Advisory Committee as Paper no. 3 of the Regional Project of the North Central States Relating to the Nutritional Status of College Women.

itself. All measurements were made by the same investigator, using the same Bausch and Lomb ocular micrometer, which had been calibrated by means of a stage micrometer. One division on the micrometer drumhead was equivalent to 0.18 micron.

TABLE 1

Summary of red cell diameter data

RANGE	MEAN IN MICRONS	NUMBER OF SUBJECTS	AGE	METHOD	INVESTIGATORS	YEAR
microns			*years*			
6.96–7.48	7.24	1 woman, 19 men	19–90	Measured 2 diameters of 500 cells in a dried stained film; projected and drawn	Price-Jones (13)	1924
Not given	7.23	20, sex not given	Not given	Measured 2 diameters of 500 cells in dried stained film	Hampson and Shackle (7)	1924
7.83–8.16	8.04	7 women,	Not given	Measured 1 diameter of 200 cells in dried unstained film	Ohno and Gisevius (11)	1925
7.74–8.03	7.88	7 men				
7.18–7.70	7.42	19, sex not given	Not given	Measured 1 diameter of 200 selected round cells in a dried film with ocular micrometer	Grosh and Stifel (4)	1925
8.55–8.60	8.56	3, sex not given	Not given	Measured 1 diameter of 100 selected cells in plasma in a photo micrograph	Dryerre, Millar, and Ponder (2)	1926
7.40–8.00	7.70	10 women, 10 men	20–30	Measured 1 diameter of 250 cells in dried stained film	Bell, Thomas, and Means (1)	1926
7.70–7.90	7.80	5 women, 3 men	Not given	Measured 1 diameter of 100 unselected cells in hirudinized plasma	Gram (3)	1927
Not given	7.32	10, sex not given	Not given	Measured 1 diameter of 200 round cells in Hayem's solution with ocular micrometer	McCormick (9)	1927
7.25–7.75	7.55	6, sex not given	Not given	Measured 1 diameter of 250 cells in dried stained film	Medearis and Minot (10)	1927
6.69–7.72	7.20	8 women, 92 men	19–90	Price-Jones technic	Price-Jones (14)	1929
8.40–8.67	8.55	5, sex not given	Not given	Measured 1 diameter of 50–100 cells photographed in serum	Ponder (12)	1930
6.70–8.00	7.50	Not given	Not given	Measured cells in dried film with ocular micrometer	Wintrobe (20)	1932
7.20–7.70	7.40	5, sex not given	Not given	Measured 200 cells in a dried stained film	Haden (6)	1934
7.02–7.25	Not given	8, sex not given	Not given	Price-Jones technic	Vaughan and Goddard (19)	1934
6.65–7.69	Not given	100 men	20–42	Price-Jones technic	Price-Jones, Vaughan and Goddard (17)	1935
6.53–7.76	7.35	80 women	17–24	Measured 1 diameter of 200 round cells in Hayem's solution, with ocular micrometer	This study	1939

Since it was thought that the use of Hayem's solution might alter somewhat the size of the cells, a comparison was made of the packed cell volume

of a series of 38 duplicate samples prepared with heparin with a similar series prepared simultaneously with Hayem's solution. The method used was that described in an earlier paper (8). Celerity in manipulation was necessary for satisfactory results with Hayem's solution, although the tubes presented difficulties in cleaning. The mean difference observed between the samples prepared with the heparin and those with Hayem's solution, −2.28 per cent, was shown to be statistically significant ($P < 0.01$) by Student's t test (18). Although it was evident from these data that the Hayem's solution did cause a small shrinkage in the volume of the cells, this effect would be appreciably less on the cell diameter. Haden (5) reported that a 13 per cent change in diameter caused a 44 per cent change in cell volume. Calculated on this basis, the observed 2.28 per cent decrease in cell volume attributable to the use of Hayem's solution would cause a decrease in cell diameter of 0.7 per cent, equivalent to a decrease of approximately 0.05 micron, an amount too small to be of any consequence.

The possibility also existed that some alteration in cell size might occur during the time required for the measurement of 200 cells. To test this possibility, the mean diameter of the first 25 of the 200 cells measured in a series of 56 determinations was compared with the mean diameter of the last 25 cells of the same series. The mean diameter of the first 25 cells was observed to be 7.17 microns as compared with a mean diameter of 7.15 microns for the last 25 cells, an obviously insignificant difference. Evidently the size of the cells does not alter during the time elapsing while measurements are made.

Results and discussion. For the series of 250 observations on 80 young women, the mean red cell diameter was 7.35 ± 0.19[3] microns; the range of mean values was from 6.53 to 7.76 microns. The median, 7.38 microns, agreed very well with the observed mean. The mean difference between the smallest and largest cells in each of these 250 blood samples was 3.56 microns, a value which closely approximated the 3.20 microns reported by McCormick (9).

Although Price-Jones (16) expressed the opinion that there was no difference in cell size due to sex, Bell, Thomas, and Means (1), who studied 20 subjects, half of whom were women, reported a mean of 7.70 microns for the women as contrasted with 7.60 microns for the men. Similarly, Ohno and Gisevius (11) gave a value of 8.04 microns for the seven women used in their investigation, as contrasted with a mean of 7.88 microns for seven men. Other investigations which included women subjects were those of Gram (3), who established a mean of 7.80 microns for a group of eight subjects, five of whom were women; and of Price-Jones (14), who reported a mean of 7.20 microns for 100 subjects, including eight women. The latter value was slightly lower than that observed in the present

[3] Standard error is used throughout in this paper.

investigation. McCormick (9), who used a method similar to the one employed in the present study, reported a mean for his 10 subjects of 7.32 microns, which was nearly identical with that secured in this laboratory. The variations in the mean diameters reported in the literature may be explained in part on the basis of the technics employed. The differences in the methods used make a comparison of the reported values difficult if not impossible, and greatly limit the usefulness of the data as a measure of the normalcy of the size of the cells in a given blood sample.

A frequency distribution based on the measurement of 20,000 cells showed a maximum spread of 3.96 to 10.80 microns, with a mean and

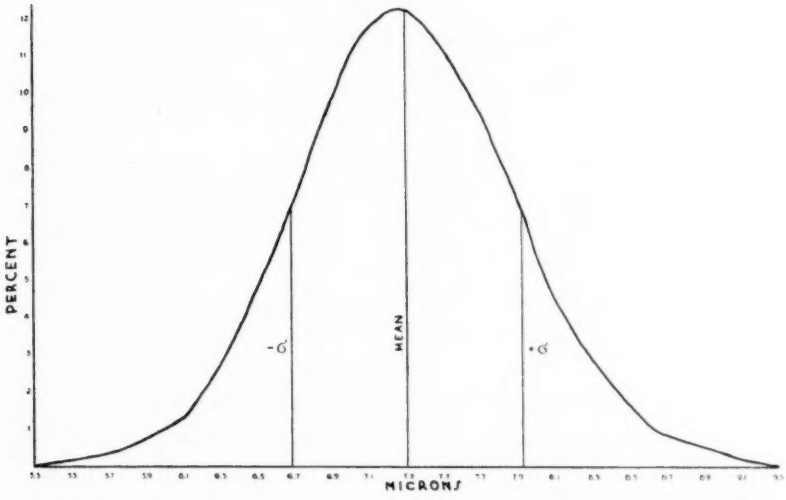

Fig. 1. Frequency distribution curve based on the measurement of 20,000 red blood cells from 100 blood samples of normal young women.

standard deviation of 7.31 and 0.62 microns respectively (fig. 1). This was a slightly greater range than that of Price-Jones (13), who, after measuring 500 cells from each of 100 persons, reported an extreme variation of 4.75 to 9.50 microns. Bell, Thomas and Means (1) noted in their study a spread in red cell diameter of 5.30 to 9.90 microns.

To determine whether there is heterogeneity in dispersion of the cells in samples with either a high or a low mean diameter, frequency distributions were made, using in one instance those items in which the mean diameter was more than one standard error greater than the mean of all, and in the other instance those items in which the mean diameter was more than one standard error smaller than the mean of all. The mean diameter

of the 41 items (8200 cells) in the large diameter group was 7.60 microns. The frequency distribution showed that the cells ranged from 4.50 for the smallest, to 1072 microns for the largest, with a standard deviation of 0.56 micron. At the other end of the scale, based on 31 items (6200 cells), the observed mean was 7.03 microns. The frequency distribution showed the range in cell size to be from 4.14 to 10.08 microns, with a standard deviation of 0.60 micron. Whether the mean diameter was large or small, the cell population was characterized by essentially the same degree of dispersion as shown by a lack of correlation between the means and the standard deviations.

TABLE 2

Red cell diameter data for the several age groups (in microns)

AGE	NUMBER OF SUBJECTS	RANGE	MEAN	STANDARD ERROR	MEDIAN
years					
17	3	6.88–7.51	7.24		7.32
18	25	6.80–7.56	7.26	0.20	7.27
19	46	7.02–7.63	7.35	0.16	7.34
20	30	7.01–7.60	7.40	0.16	7.42
21	12	7.05–7.67	7.20	0.20	7.20
22	10	6.69–7.62	7.30	0.28	7.30
23	4	7.20–7.46	7.36		7.40
24	1		7.31		7.31

TABLE 3

Analysis of variance of red cell diameter with age

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN OF SQUARES
Total	130	
Between age groups	3	0.13*
Between individuals of same age	127	0.01

* Highly significant.

In view of the relatively wide distribution in cell size in a given sample, it was interesting to note the minimum and maximum values in this series. The mean of the smallest cell in each of 250 determinations was 5.52 microns, and of the largest cell, 9.08 microns.

Table 2 was prepared to show the mean cell diameter values secured for the various age groups. An analysis of variance computation showed that there was a highly significant difference between the mean cell diameters of the various age groups. For the purposes of this calculation, the 17- and 18-year-olds were grouped together, as were those 22 years of age and older. Table 3 gives the results of this computation.

As a further investigation of the effect of age on cell size, data were secured a year apart on 28 persons. These showed a mean increment in red cell diameter of 0.20 micron between the two points of time. To demonstrate that the increase in cell size from year to year was real and not due to within-individual variations, an analysis of variance computation was carried out on the data of 13 of the above 28 subjects on whom *duplicate* determinations were made at the time of the two analyses. The results of this computation are shown in table 4, from which it is evident that the variation from year to year was greater than the within-individual

TABLE 4

Analysis of variance of yearly increment in red cell diameter

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN OF SQUARES
Total	51	
Within—individuals	26	0.30
Individuals	12	1.49*
Time (year)	1	14.25*
Interaction	12	0.43

* Highly significant.

TABLE 5

Analysis of variance of seasonal increment in red cell diameter

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN OF SQUARES
Total	67	
Within—individuals	34	0.36
Individuals	16	1.58†
Time (seasons)	1	2.43*
Interaction	16	0.68

* Significant.

† Highly significant.

variation. It was clear, therefore, that a definite increase occurred in the mean diameter of the cells of these young women in this time interval.

It seemed important also to ascertain whether a greater portion of this increase occurred during the summer than during the winter months. There were available diameter determinations on a considerable number of subjects, spaced at six-month intervals; i.e., there were 31 persons on whom determinations had been made during the first spring the present study was in progress and again during the following fall; a second group of 19, on whom determinations had been made in the fall and again in the succeeding spring; and a third group of 17, on whom determinations had been made during the second spring of the study, and again in the fall. The increases

from spring to fall, and from fall to spring, were of the same magnitude, the mean increase in cell diameter in the first group being 0.11 micron; in the second group, 0.12 micron; and in the third group, 0.13 micron.

To determine whether the variation from season to season was significantly greater than the within-individual variation, analysis of variance was computed on the data from 17 subjects on whom duplicate determinations had been made six months apart. From the results recorded in table 5 it is evident that the observed difference in cell diameter due to season is greater than the within-individual variation.

The only study which the authors were able to locate in the literature which recorded observations made at intervals on the same subject was that of Price-Jones (15), who found no significant change in the cell diameters of two subjects after a period of 12 and 3 months, respectively. Two additional subjects on whom he made a second measurement after an interval of 10 years also showed no change in cell diameter. These findings cannot be interpreted in the light of the changes noted in the present study, since no ages were given for his subjects.

In the present investigation it was apparent that a significant increase in the diameter of the red blood cells occurred with increase in age. This change may be concomitant with the physiological maturation of young women of this age range; or it may be a reflection of an improved environment.

The extent of daily variation in the cell diameter of a middle-aged woman was demonstrated by a series of 27 determinations made within a period of 32 days. The mean diameter for the entire series was 7.06 microns; the range, 6.91 to 7.25 microns. Computation of the differentiation of the daily means showed that the variations in the mean diameter of the erythrocytes that occurred from day to day were statistically significant.

Price-Jones, Vaughan, and Goddard (17), in a study on one subject on whom observations were made over a two-months' interval, noted similar variations in the daily means, the range reported being from 6.96 to 7.18 microns.

It is anticipated that the values presented in this paper will be useful in augmenting the information on an age group for which few data are available.

SUMMARY

1. Red cell diameter measurements were made on 250 blood samples taken from 80 healthy college women, ranging in age from 17 to 24 years. The mean diameter was 7.35 microns; the range of means from 6.53 to 7.76 microns. The mean difference between the smallest and largest cells in each of these blood samples was 3.56 microns.

2. A frequency distribution, based on the measurement of 20,000 cells,

showed a maximum spread of 3.96 to 10.80 microns, with a mean and standard deviation of 7.31 and 0.62 micron respectively.

3. The dispersion in the red cell population was of essentially the same magnitude in those blood samples for which a high mean diameter was secured as in those for which a low mean diameter was obtained.

4. The means of the values for the smallest and for the largest cell in each of 250 determinations were 5.52 and 9.08 microns, respectively.

5. Observations spaced a year apart on 28 individuals showed a mean increment of 0.20 micron, distributed equally between the summer and winter semesters. This increase proved to be statistically significant.

6. Twenty-seven observations on a middle-aged subject over a 32-day period yielded a range of 6.91 to 7.25 microns, and a mean diameter of 7.06 microns.

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THE RELATION OF ELECTRICAL AND MECHANICAL EVENTS IN THE VENTRICLE OF THE TURTLE[1]

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For many years, the assumption has been made that the spread of activity over heart muscle is accompanied in time and in place by an uncomplicated fall in electrical potential, as a result of which the active muscle becomes electro negative with respect to resting muscle. This conception, originating from certain early experimental results obtained in studies on the electrophysiology of nerve, has not until recently been subjected to adequate experimental criticism. By recording the electrical potentials of heart and skeletal muscle under experimental conditions which permit accurate analysis of the situation to be made, it has been clearly shown that during activity there are regions in which the potential rises above, as well as regions in which it falls below, the potential of the tissue when at rest.[2] The electrical phenomena which accompany activity are thus more complicated than postulated by the "negativity theory" and it is not possible at present on theoretical grounds to define the relationship which presumably exists between the electrical and contraction processes in muscle. The problem is one which can only be solved by experimentation.

Previous work reported from this laboratory (1), in which the potential distribution in a conducting field surrounding the turtle's heart was determined, proved without question the polar nature of the electrical charge distribution in the heart. The nature of the experiments was such, however, that they did not reveal in detail the location nor the number of regions in which positive or negative potentials occurred during the period of activity of the ventricle. An answer to the latter question was the purpose of the first series of experiments to be reported in the present communication. Having established in detail the electrical pattern associated with activity, a second series of experiments was carried out in which the relative times of onset of contraction in localized regions of the ventricular surface were determined in an effort to relate the electrical and

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[2] For literature, see (1).

mechanical patterns. Finally, two types of potential-time curves, unipolar and differential, were recorded from various regions of the ventricular surface and studied with reference to each other and to the onset of the contraction process.

METHODS. All experiments were made on the hearts of large specimens of the snapping turtle (Chelydra serpentina) in situ, or isolated at the center of a large circular conducting field. Recordings of mechanical and electrical events were made by the aid of direct current amplifiers (2) and cathode ray oscillographs on bromide paper. Time intervals on records were measured by means of a magnifying comparator of high accuracy (3).

Electrical field on the surface of the ventricle. Unipolar potential time curves were recorded from 40 different points on the surface of the ventricle simultaneously with a reference curve derived from a fixed point on the ventricle, or from the field surrounding it. The electrode used as the distal lead for the unipolar records was applied to a hind leg in experiments with the heart in situ, or to a distant point in the field in the case of the isolated heart. The period on the unipolar curves corresponding to the QRS interval was divided into 14 equally spaced time instants. For each of these instants the potentials at each of the 40 points on the ventricle were determined by measurement of the calibrated records, the potential of resting heart muscle being taken as the zero of reference. The values thus obtained were entered on separate drawings of the ventricular surface at each of the 40 points and equipotential lines drawn.

Contraction in localized regions of ventricular muscle. For recording contraction in localized regions an electrical myocardiograph was devised, consisting of two celluloid strips, 1 by 6 cm., joined at the top by means of a flexible cloth joint to form a narrow inverted V and between these there is mounted a strip of paper folded in the form of a V. When in use, the paper is wet with weak salt solution and connected in series with an oscillator delivering an alternating current of twenty thousand cycles per second. The approximation of the open ends of the celluloid V shortens the effective electrical path over the wet paper and results in a decrease of electrical resistance. The alternating current, employed to avoid polarization in the paper wet with electrolyte, is rectified and the voltage changes resulting from changes in resistance are amplified and recorded. The open ends of the myocardiograph, to which short blunt wires are cemented to prevent slippage on the heart surface, are applied to a local region of the ventricle with the ends 2 or 3 mm. apart. The device weighs about a gram and very slight pressure is sufficient to ensure effective application to a small region of the ventricular surface. The additional load applied to the muscle is slight and there is no evidence of injury. If care is used to place the open leaves in the general direction in which the muscle shortens, uniform curves

are obtained with the onset of contraction, in most cases, sharply marked. It is possible to secure records from 16 or more separate regions on the large ventricle of the snapping turtle. To determine the relative time of onset of contraction at different regions, a reference electrogram, usually a unipolar lead from the ventricular apex, is simultaneously recorded.

Unipolar and differential potential-time curves. In experiments in which these curves were recorded simultaneously, two zinc chloride electrodes provided with a common wick were employed. The center of the wick, compressed to a V shape, was applied to the ventricular surface. Leads from the two electrodes to one amplifier served for the differential record; one of the electrodes connected to a second amplifier along with a lead from a distant point in the animal, provided for the unipolar record. In experiments in which differential electrode and contraction curves were recorded simultaneously, the myocardiograph was provided with two electrodes at its open ends to serve as a combined myocardiograph and differential electrode.

RESULTS. *The potential field of the ventricular surface.* Sixteen drawings of equipotential lines, derived in the manner described from a typical experiment, are reproduced in figure 1. The instant in the QRS period represented by each of the plots is indicated by the line drawn through the reproduction of the reference curve immediately beneath each plot. Negative areas are shaded, positive areas unshaded. Regions of relative maximum potential are indicated by plus and minus signs and represent sources and sinks respectively. The drawings shown are for the anterior surface of the ventricle. The distribution on the posterior surface is a mirror image of these.

The first indication of electrical activity is signalled by the simultaneous appearance of a sink, or negatively charged region, at the right base, and two sources, or positively charged regions, one near the left base, the other near the apex. The potentials of all are low at the start, a half mv. or less. In the succeeding several instants, all the potentials grow, to reach -3.5, $+5.0$ and $+3.1$ mvs., respectively. There is also an extension downward and to the left of the negatively charged region. In the sixth drawing a new phase is introduced, consisting in the development of a new sink along the left side of the great vessels which grows rapidly in the extent of area involved and in magnitude, attaining a value of -24 mvs. coincident with the peak of the reference curve. The initial sink at the upper right base simultaneously declines and is replaced by a region of positive charge. At this stage three sources are present, of which the two larger ones are to the right of the mid-ventricle ($+8$ mvs.) and near the apex ($+6$ mvs.). The initial source at the left base declines rapidly and soon disappears. There thus results a fairly rapid shift of charge orientation, beginning after the completion of about one-fourth of the QRS period, and the direction of

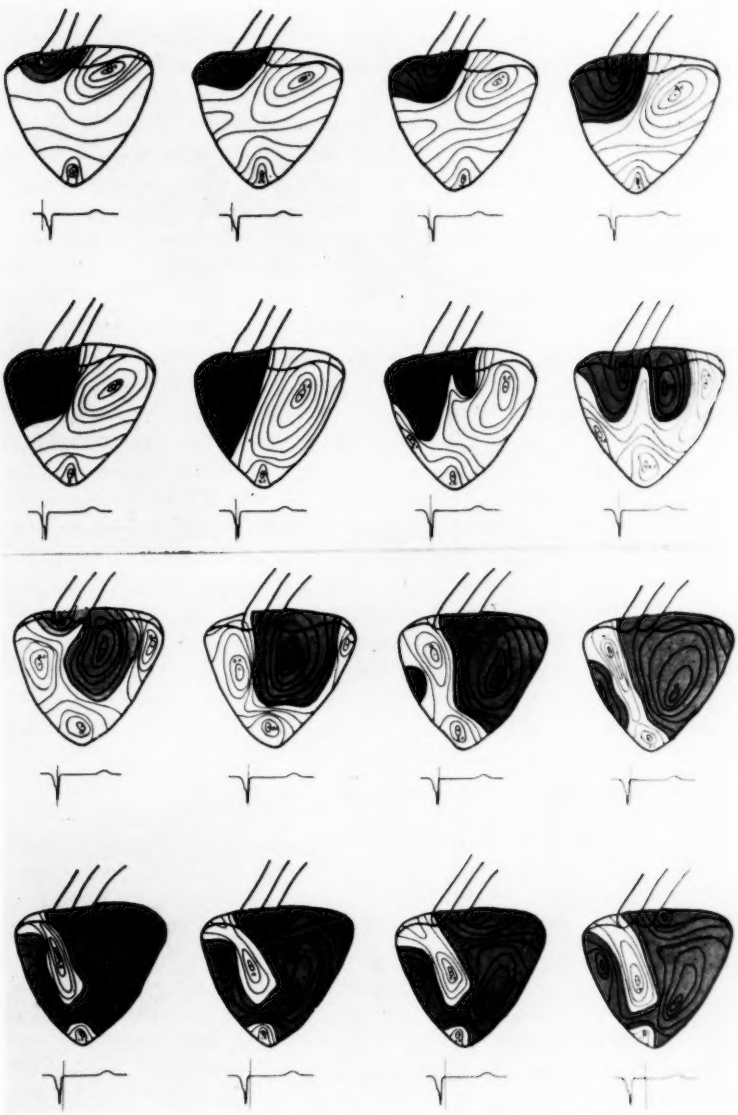

Fig. 1. Equipotential lines of the electric field of the turtle's ventricle. For description see text.

electrical intensity changes through an angle of approximately 90 degrees. This shift was observed previously in potential studies in a conducting field around the heart (1), and accounts for the disappearance of the first, and the development of the second dipole, of the QRS period. Accompanying the change in orientation, the negatively charged region increases in size and the positive charges become concentrated in two relatively small regions. In the upper one of these, extending from near the mid-ventricle to the right base, the potential rises to 30 mvs., the maximum potential attained at any time in any region. All potentials finally decline rapidly to values of less than 1 mv. during the so-called iso-electric period.

The potential field on the isolated heart and on the heart in situ showed no essential differences.

Sequence of contraction at different regions on the anterior surface of the ventricle. The usual sequence of onset of contraction of 16 regions on the anterior surface of the ventricle derived from 12 experiments is given in figure 2. Certain differences in detail were encountered in some of the experiments; but in all, the left base or the immediately contiguous region was the first part involved. Thus in 6 experiments, region 1 was the first region to enter into contraction, while initial activity occurred at region 2 in 4 cases and at region 3 in the remainder. The mid-region of the ventricle contracts next, and finally, the apex and right base are involved. The average interval between the region first to contract and the region last to contract in the 12 experiments was 0.1345 sec., with extremes of 0.0816 sec. and 0.1887 sec. Rather rapid involvement of separate regions on the left base occurred with intervals usually of only a few hundredths of a second, while larger intervals separated the involvement of regions near the apex and right base. In figure 3 curves are shown from two regions, one entering into contraction early, the other late, in the sequence.

Relation of unipolar and differential curves to each other and to the onset of contraction. A number of preliminary experiments were done in which differential curves or unipolar curves were recorded simultaneously with a reference curve; the latter, in most cases, being a unipolar curve derived from the ventricular apex and leg. The results from these experiments showed that the sequence of occurrence of the main peak of differential curves followed closely the sequence of involvement of the ventricle in contraction as determined previously. The earliest main peaks occurred in records from the left base and contiguous regions, while at the apex and right base, the differential peaks occurred considerably later. The correspondence was such as to suggest a close relation between the peak of the principal deflection of the differential curve and the onset of contraction. Similar experiments, in which unipolar curves were recorded, showed much less similarity of pattern. From most regions on the ventricle, the unipolar curves are diphasic and neither one of the peaks showed any consistent relation to the onset of contraction.

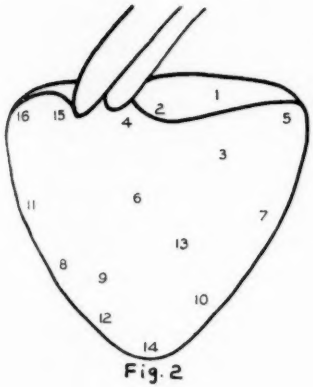

Fig. 2

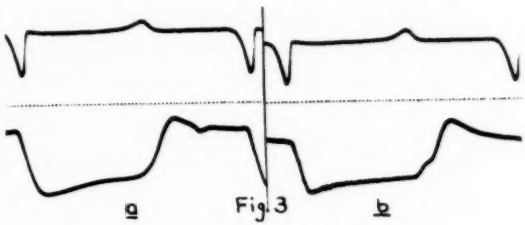

Fig. 3

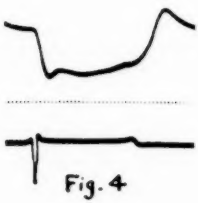

Fig. 4

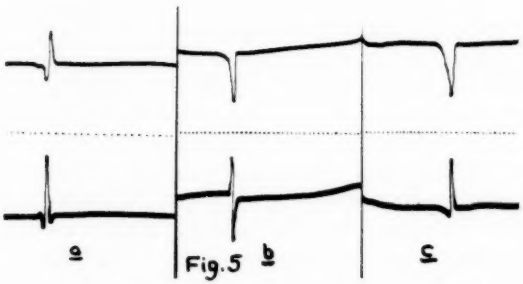

Fig. 5

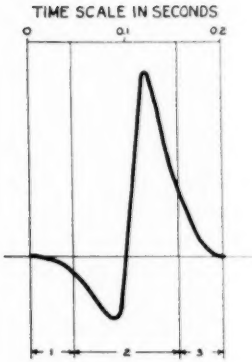

Fig. 6

Fig. 2. Sequence of onset of contraction in different regions of the anterior surface of the turtle ventricle. For further description see text.

Fig. 3. Onset of contraction in two different regions of the turtle ventricle. Upper curve in each case is a unipolar curve from the apex, lower curve records local contraction. In record *a*, the myocardiograph was on region 1 of figure 2. Onset of contraction precedes peak of reference curve by 0.0786 sec. In record *b*, the myocardiograph was on region 12. Onset of contraction follows reference peak by 0.0181 sec. Difference between time of onset of contraction of the two regions is therefore 0.0967 sec.

Fig. 4. Simultaneous recording of contraction (upper curve) and differential potential time curve (lower curve) from a localized region of the surface of the turtle ventricle. The start of the down stroke of the contraction curve is coincident, within the errors of measurement, with the main peak of the differential curve. Time in 0.04 sec. intervals.

Fig. 5. Simultaneous recordings of unipolar curve (upper curve) and differential potential curve (lower curve) from three separate spots on the turtle ventricle. For description see text.

Fig. 6. The three phases of electrical activity during the QRS period. The curve is reproduced from a unipolar potential curve recorded from a point on the surface of the turtle ventricle.

Simultaneous recording of contraction and of differential or unipolar curves was now carried out. In 9 experiments, comprising a survey of 76 points, there was found a close correspondence between the occurrence of the main peak of the differential curve and the onset of contraction at different regions. In many cases, the two were simultaneous within the errors of measurement and in the large majority of instances, the interval between the two was less than 0.01 sec. In 3 of these experiments in which records were made from 34 separate points using the combined myocardiograph and differential electrode, the main peak of the differential curve preceded the onset of contraction by an average of 0.0023 sec. with extreme variations from this mean of +0.0178 and −0.0110 sec. The standard deviation of the distribution was ±0.0081 sec. An example of the records obtained in these experiments is given in figure 4. When it is considered that the accuracy of measurement is reduced by the fact that the onset of contraction, while quite sharp, cannot be as accurately placed as the extremely sharp peaks of unipolar or differential records, the correspondence is evident. Extreme variations of the relative time of onset of contraction or of differential peaks at *different* regions are of the order of 0.14 sec.

In 6 experiments in which unipolar and contraction curves were recorded simultaneously, it was evident that there was no close correspondence between the onset of contraction and either of the two peaks of the usual diphasic curve or of the peak of the less frequent monophasic curve. Contraction appeared to start in the large majority of cases in the period of about 0.04 sec. lying between the two peaks of the diphasic curve, or on the ascending or descending limb of the monophasic curve. The true relation was disclosed by recording differential and unipolar curves simultaneously. In two experiments of this sort, in which recordings were made from 30 separate points, the main peak of the differential curve coincided in all cases with that part of the unipolar curve in which the potential was changing most rapidly, i.e., during the period of maximum gradient. From 21 of the 30 points the unipolar curves were diphasic, and in 19 of these, the main peak of the differential curve occurred between the two (positive and negative) peaks of the unipolar curve. In 2, it fell on the distal limb beyond the second peak. In 9 of the 30 records the unipolar curves were monophasic or nearly so. In these the main differential peak fell on the proximal limb in 2, and on the distal limb in 7. Three examples of these curves are shown in figure 5. In record *a*, the unipolar curve is diphasic and the main differential peak falls in the region of maximum gradient between the positive and negative peaks of the unipolar curve. There are minor peaks on the differential curve coinciding with the regions of less steep gradient preceding the first and following the second peaks of the unipolar curve. In record *b*, the unipolar curve is monophasic and the proximal limb is somewhat less steep than the distal limb. This is

reflected in the differential curve by two peaks, the larger one coinciding with the steeper gradient following the peak of the unipolar curve. In *c*, the unipolar curve is again monophasic and the gradient on the distal limb is much greater than that on the proximal limb. Two peaks occur on the differential curve, a small one falling on the initial gradual gradient and a very much larger one falling along the steep gradient of the distal limb of the unipolar curve.

Discussion. Contraction of all points of the ventricular surface is preceded by the development of an electrical potential field. This field is characterized by the simultaneous appearance of sources and sinks, which are at first more or less stationary and grow to a relatively small magnitude (4 to 6 mvs.) over a period of approximately 0.05 sec. This period may be defined as the first phase of electrical involvement. A second phase now occurs which is characterized by a simultaneous decline of the initial sources and sinks and the appearance of new sources and sinks with different orientation than that of the first. These rise to a considerably higher potential, to a maximum of 30 mvs., and change somewhat in position as growth proceeds. It is during this second phase, which has a duration of about 0.13 sec., that the different regions of ventricular muscle enter into contraction. A final or third phase is characterized by a decline of the sources and sinks to reach a nearly iso-potential state over a period of about 0.05 sec. During this phase little or no change in orientation of charges occurs. The approximate relation of these phases to the QRS period is indicated in figure 6. Referring to the electrical field as illustrated in figure 1, the first 6 drawings would fall in the first phase, the next 6 in the second, and the remaining drawings in the final phase.

Comparison of the electrical field at the different instants during the QRS period would indicate that the occurrence of contraction at any given region is not associated with the occurrence of a source or sink at this region, but with potential gradients established between neighboring sources and sinks. That the latter situation is the true one is shown by the analysis of results from unipolar and differential potential curves.

In this connection it is important to have a clear conception of the electrical significance of these curves. Unipolar curves are obtained by applying one electrode to the ventricular surface and the other to a region sufficiently far from the heart so that it is little affected by electrical changes occurring at the heart. A unipolar curve is a record of the potential change at a given spot relative to the potential of resting heart muscle. The peaks represent the maximum potentials, positive or negative, that occur at this spot. The rate of change of the curve, or time gradient, represents the rate at which the potential is changing.

The differential curve obtained by means of two electrodes placed close together on the ventricular surface, is a measure of the potential gradient, or current flow, at the spot. Peaks represent periods of maximum current

flow. The time rate of change of the curve represents the rate of change of current flow. During the brief duration of the peaks, the rate of change of current flow is zero, but extremely rapid changes immediately precede and follow the main peak.

From the foregoing description, it is evident that mechanical activity, represented by the onset of contraction, occurs coincident with, or is separated by a very brief interval from, the establishment of the maximum electrical potential gradient, the maximum flow of electrical current, and the most rapid rate of change of current flow in this region. This period is coincident with the main peak of the differential curve and with the maximum time gradient of the unipolar curve.

The association of contraction with the optimum conditions for electrical stimulation, maximum flow of electricity and maximum rate of change of electrical current, suggests the possibility that the normal stimulus to contraction in different regions of the ventricle is electrical. Our present knowledge, however, is not sufficient to indicate whether or not this relation actually exists.

If a period of isometric contraction exists in the turtle's heart, it appears to exert no effect on local contractions. We have carried out a number of experiments on the bloodless heart and the same relations were found between the unipolar and differential curves and between these curves and the onset of contraction as in the blood-containing heart.

CONCLUSIONS

The potential field on the surface of the ventricle of the snapping turtle during the QRS period is characterized by the simultaneous appearance of electrical sources and sinks, or regions of positive and negative charges. The sources and sinks show periods of growth and decline and changes in orientation. The initial electrical manifestation precedes by about 0.05 sec. the appearance of contraction in any surface region of the ventricle, and persists for an equivalent period following the last region to enter into contraction.

The onset of contraction at various regions of the ventricular surface is coincident with, or separated by a very brief interval from, the occurrence of maximum electrical current flow across the region and the maximum time rate of change of this current. This electrical state coincides with the main deflection of the differential curve and the most rapid rate of potential change in the unipolar curve recorded from this region.

The association of contraction with optimal conditions for electrical stimulation suggests, but does not prove, that the normal stimulus to contraction is electrical.

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THE MECHANISM OF THE INHIBITING EFFECT OF ELECTROLYTES AND HEPARIN ON BLOOD COAGULATION

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The addition of neutral salts to blood will slow down the rate of coagulation and, at definite concentrations, will prevent clot formation (2) but the mechanism responsible for this effect is not known. The anticoagulant effect of salts has been reported to increase with the valency of the ions (10, 15, 20), although an accelerating effect has been observed for very low concentrations of certain heavy metal cations (8). Mercury and silver were particularly effective. Coagulation is accelerated by small amounts of calcium salts due to the specific rôle of ionic calcium in thrombin formation (6), but an excess of calcium salts will inhibit coagulation (12). Cations appear to be more effective than anions in preventing coagulation, judging by the concentrations of polyvalent ions required to prevent coagulation. Häusler and co-workers (10, 11) studied the inhibiting effect of polyvalent cations and the effect of the fluoride, oxalate, and citrate of sodium on calcium-free reagents. Other observations have been reported for the anticoagulant action of ferrous sulfate, ferric phosphate, and potassium ferrocyanide (1), but no systematic study of the effect of electrolytes on coagulation has been undertaken.

The results reported in this paper were obtained by studying the effect of various ions on the coagulation of fibrinogen by thrombin. They indicate that anions act directly on thrombin, while cations inhibit coagulation by acting on fibrinogen.

EXPERIMENTAL PROCEDURES. *Reagents.* The following reagents were used in the experimental work:

1. *Plasma.* Freshly drawn beef blood to which 0.1 per cent of potassium oxalate had been added was centrifuged at high speed to throw down the formed elements. The supernatant plasma was then drawn off and recentrifuged.

2. *Diluted plasma.* This reagent was used as a source of fibrinogen in certain experiments. To the plasma obtained as described above there

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were added three volumes of an 0.85 per cent NaCl solution which contained a trace of oxalate. The amount of oxalate present was sufficient to precipitate the calcium present in any thrombin preparation added to the plasma during the course of the experiments, thereby preventing activation of the prothrombin. The purpose of the dilution was to minimize the effect of any antithrombic substances which may have been present.

3. *Fibrinogen.* The method of purification used was essentially the same as described by Eagle (5), in which NaCl was used to salt out the fibrinogen.

4. *Thrombin.* Ten milliliters of plasma were diluted with 100 ml. of distilled water, and CO_2 was bubbled through for 5 minutes to saturate the solution. The resulting globulin precipitate was removed by centrifugation and dissolved in 10 ml. of saline solution to which was added a little $NaHCO_3$ to bring the pH to about 7.0. The mixture was warmed to 38° and to it was added 0.5 ml. of a thromboplastin solution (such as heated saliva (9) or a saline extract of brain), and 0.5 ml. of a 0.1 M $CaCl_2$ solution. The fibrin which appeared was removed with a stirring rod as fast as it formed. The residual solution had a high degree of thrombic activity.

Technic. The effect of electrolytes on the coagulation of fibrinogen by thrombin was studied by changing the concentrations of each of the three reagents while the other two were held constant. The technic used was to add a measured volume of the electrolyte solution to a constant quantity of fibrinogen solution or diluted plasma contained in a 10 x 75 mm. serological tube, and then sufficient physiological saline was added to bring the final volume to 0.8 ml. The mixture was heated to 38° in a thermo regulated water bath. Then 0.2 ml. of the thrombin solution was added and the coagulation time noted by tilting the tube every few seconds until the mixture ceased to flow. The electrolyte concentrations given in the tables and figures refer to the concentrations present after the addition of the thrombin solution.

RESULTS. *Electrolytes.* Using a fixed concentration of fibrinogen and thrombin, a series of values for the coagulation time was obtained with different concentrations of electrolytes as indicated in figure 1. The salts used were chosen to obtain different values for the valency of the anions, the cations all being univalent. It will be noticed that the degree of inhibition increased markedly with the valency of the anions studied. The dipolar ion compounds, urea, glycine and cystine,[2] showed no appreciable inhibitory effect, but cysteine exhibited a marked inhibitory action. The presence of the free —SH groups in cysteine probably caused this effect

[2] The data for cystine are not plotted in figure 1.

by acting through denaturation of the proteins. Sterner and Medes (19) demonstrated an anti-prothrombic action for cysteine in concentrations greater than tenth-molal, but the present results clearly indicate that cysteine also has an anti-thrombic action.

Upon using solutions of equivalent ionic strength (14), the same degree of inhibition was obtained with the oxalates, ferricyanides, and ferrocyanides, but not with the salts of other anions. Therefore the inhibiting effect of anions is not solely a function of the ionic strength of the solutions. The effect of anions on the second phase of coagulation is negligible for univalent ions, and increases strongly with the valency.

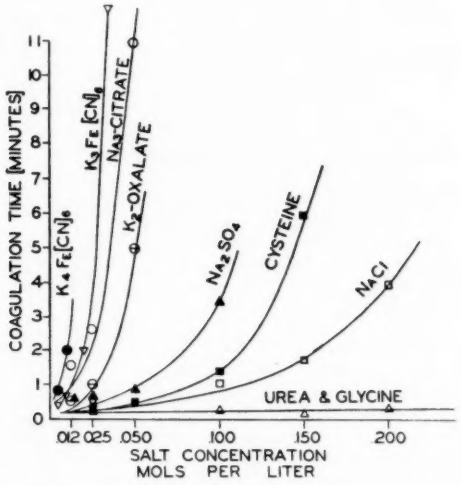

Fig. 1. Inhibiting effect of electrolytes and dipolar ion compounds on the second phase of blood coagulation.

Similarly, the effect of cations on coagulation was found to be negligible for ions of low valence, but increased markedly for those of higher valence. Thorium salts were found to be very effective in retarding the rate of coagulation. In one experiment, for example, 0.0001 M $Th(NO_3)_4$ was found to prevent coagulation completely, while the fibrinogen clotted 60 seconds after the addition of thrombin when 0.1 M $MgCl_2$ was present. Of course the effect of the ion being studied may be modified by the presence of the accompanying oppositely charged ion; but since the anticoagulant effect is increased so markedly by an increase in valency, the results obtained with uni-tetravalent salts, for example, may be attributed entirely to the polyvalent ions.

To study this effect further, the coagulation time was measured using

different concentrations of thrombin and fibrinogen. The results obtained by using potassium ferrocyanide and different amounts of thrombin are shown in table 1. The data of table 1 (series I) have been plotted in figure 2. The amount of thrombin used to coagulate the fibrinogen makes a great difference in the apparent effect of the polyvalent anions. It is obvious that the inhibitory effect of the ferrocyanide ion is overcome by the addition of thrombin, and when more thrombin is used, more of the ferrocyanide must be added to obtain the same degree of inhibition. The concentration of ferrocyanide ions required to produce a given degree of

TABLE 1

Relation between thrombin concentration and the inhibiting effect of ferrocyanide ions on blood coagulation

$K_4Fe(CN)_6$	RELATIVE AMOUNTS OF THROMBIN (COAGULATION TIME GIVEN IN SECONDS)				
	2	4	6	8	10
Series I					
mols. per liter					
0	15	10	7	6	5
0.01	70	27	25	20	18
0.02	600	80	55	55	40
0.03			130	80	65
0.06					105
Series II					
0	11	10	8	9	8
0.005	35	27	20	17	11
0.010	53	40	25	22	21
0.015	70	55	43	37	32

TABLE 2

Relation between the fibrinogen concentration and the inhibiting effect of ferrocyanide ions on blood coagulation

$K_4Fe(CN)_6$	RELATIVE CONCENTRATIONS OF FIBRINOGEN (COAGULATION TIME GIVEN IN SECONDS)	
	1	2
mols. per liter		
0	6	6
0.005	13	11
0.010	20	16
0.015	28	27
0.025	55	60

increase in the coagulation time appears to be dependent upon the thrombin content.

Similar experiments in which the thrombin concentration was kept constant while the concentration of fibrinogen was varied had very little effect on the coagulation time, thus showing that the fibrinogen concentration was not important in the anticoagulant action of polyvalent anions. These results are given in table 2.

Analogous experiments were carried out using thorium nitrate instead of the ferrocyanide salt. Table 3 shows the results of a typical experiment in which the concentration of thrombin was kept constant.

It will be seen that the inhibiting action of the thorium salt was not pronounced until after a definite concentration was reached, beyond which a modified form of fibrinogen or fibrin came out of solution when thrombin was added. At higher concentrations, fibrinogen flocculated out of solution without the necessity of having thrombin present. The amount of thorium salts required to produce these effects apparently depended upon the concentration of fibrinogen that was present. On the other hand, increasing the amount of added thrombin had very little effect on the coagulation time. This is shown in table 4. Thus the primary action of

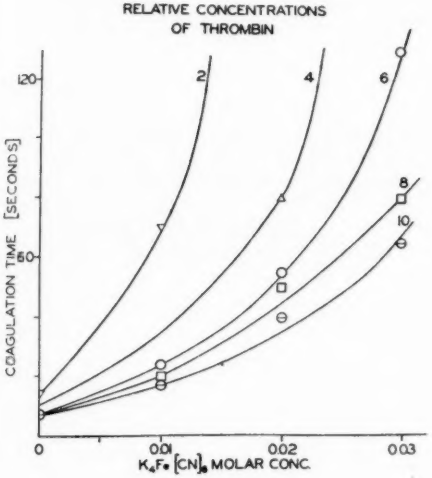

Fig. 2

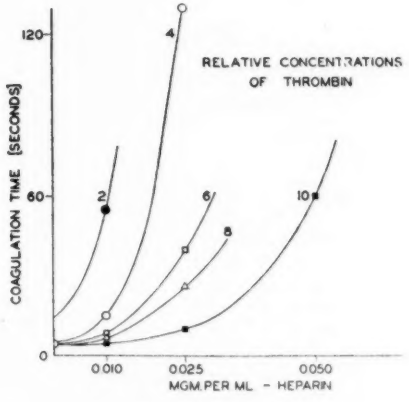

Fig. 3

Fig. 2. Antagonistic effect of thrombin on the inhibiting action of ferrocyanide ions on blood coagulation.

Fig. 3. Antagonistic effect of thrombin on the inhibition of blood coagulation by heparin.

the thorium cations appears to be on the fibrinogen, which is in accord with the findings of Häusler and Vogel (11).

Heparin. Fischer (7, 8) demonstrated that the addition of heparin to certain plasma proteins resulted in a shift of the isoelectric point of the proteins toward the acid side. This property of heparin afforded a clue to the mechanism of its antithrombic action, since the number of negative groups on the protein molecules would be increased at the pH of blood, possibly resulting in the same type of behavior as exhibited by inorganic polyvalent anions. Heparin has no anticoagulant action whatsoever when purified clotting reagents are used (13, 17), but is effective in the presence of plasma. Quick (17) has indicated that the factor in plasma responsible

for this behavior is associated with the albumin fraction. Using various concentrations of heparin in the presence of a constant amount of diluted plasma, to which different concentrations of thrombin had been added, gave the series of results shown in figure 3.

These results are strictly analogous to those obtained with the ferrocyanide anions, as may be seen by comparing figures 2 and 3. The mechanism of the inhibiting action is probably the same. Heparin has been shown to be a strongly sulfonated compound, and its anticoagulant activity has been attributed to the presence of these groups, inasmuch as synthetic sulfonated compounds have been developed which are active anticoagulants (3). The anticoagulant activity of heparin is roughly a thousand-fold greater than that of the ferrocyanide ion, presumably because it has a higher density of electrical charge.

TABLE 3

Relation between fibrinogen concentration and the inhibiting effect of thorium ions on blood coagulation

$Th(NO_3)_4$	RELATIVE CONCENTRATIONS OF FIBRINOGEN (COAGULATION TIME GIVEN IN SECONDS)		
	1	2	3
mols per liter $\times 10^{-6}$			
0	13	13	14
3.5	13	13	
17.5	14	11	
35.0	20*	14	9
70.0	35*	15	14
140.0	5*	20*	17
350.0	†	8*	†
700.0	†	†	†

* Instead of a clear gel, strands of protein appeared in the time indicated after the addition of thrombin.

† The fibrinogen flocculated from the solution immediately on addition of the specified amount of $Th(NO_3)_4$, and *before* the addition of thrombin.

TABLE 4

Relation between thrombin concentration and the inhibiting effect of thorium ions on blood coagulation

$Th(NO_3)_4$	RELATIVE AMOUNTS OF THROMBIN ADDED TO DILUTED PLASMA (COAGULATION TIME GIVEN IN SECONDS)		
	1	3	5
mols per liter $\times 10^{-6}$			
0	12	4	4
7	13	5	4
70	13	5	4
140	12	5	4
350		6	5

Effect of pH. Another set of observations on the effect of the electrical charge on coagulation was obtained from a study of the effect of the hydrogen ion concentration on the isolated phases of coagulation involving 1, the formation of thrombin, and 2, the action of thrombin on fibrinogen. Figures 4 and 5 demonstrate the results of these experiments. The rea-

gents and technic used in studying the rate of thrombin formation were described in a previous publication (9). The pH measurements were obtained with a glass electrode.

The optimum pH for the formation of thrombin lies between about pH 6.5 and 7.5, that for the production of fibrin by thrombin between about pH 6.0 to 8.0. Both processes are rapidly inhibited as the hydroxyl ion concentration increases. In the light of the discussion to follow, a probable explanation of the inhibition of fibrin formation by alkali is that the hydroxyl ions shift the charge of the thrombin towards a negative value, perhaps actually reversing its positive charge, thereby preventing combination with the negatively charged fibrinogen substrate.

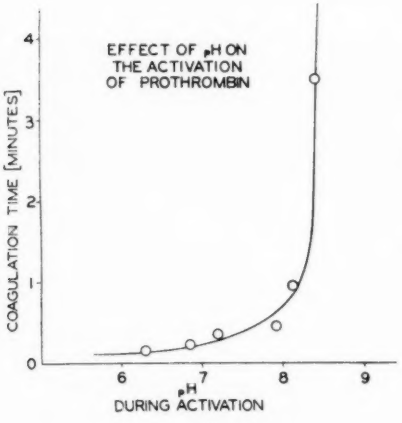

Fig. 4

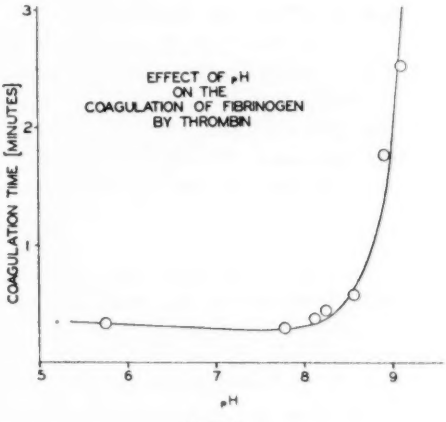

Fig. 5

Fig. 4. Effect of pH on the activation of prothrombin
Fig. 5. Effect of pH on the coagulation of fibrinogen by thrombin

The effect of pH on the activation of prothrombin is more difficult to interpret because the electrochemical properties of thromboplastin are not established. However, it seems likely that the influence of the ionic charges on the formation of an enzyme-substrate intermediate is involved.

Discussion. A tentative explanation of the mechanism of the inhibiting action of the charged molecules or ions on coagulation is presented here in an attempt to correlate these results. At the present time there is fairly good evidence for the enzymatic nature of thrombin (16). The formation of an enzyme-substrate complex prior to any chemical change in the substrate is in accord with modern concepts of enzyme chemistry. Fibrinogen is negatively charged at the pH of blood, since its isoelectric point lies between 5.2–5.6 (18). Thrombin is probably a protein also, and there is some evidence that it is positively charged at the pH of blood (4). The

combination of the two reagents is at least partly dependent upon their potentials which may be altered in the presence of electrolytes. For example, the presence of polyvalent anions would repress the ionization of the basic groups on the thrombin molecules, resulting in a diminution of the net positive charge on the thrombin and a consequent decrease in the amount that could combine with fibrinogen. This, in turn, would decrease the rate of coagulation, since the concentration of the intermediate enzyme-substrate complex would be reduced. It has been shown that the action of both heparin and polyvalent anions is directly on thrombin, and it is very likely that the mechanism of the "antithrombic" action of heparin is the same as that for polyvalent anions. A similar situation exists for the interaction of fibrinogen with polyvalent cations so that the potential becomes less negative.

The alteration of the potentials of either the thrombin or fibrinogen by polyvalent anions or cations, respectively, because of interference with the formation of an enzyme-substrate intermediate through combination of the positively and negatively charged proteins, inhibits the rate of fibrin gel formation. The action of the hydrogen ion concentration on coagulation has already been explained above along similar lines.

SUMMARY

1. A study has been made of the inhibiting effect of various electrolytes and of heparin on the second phase of coagulation. The potency of the electrolytes was found to increase with the valency, but not as a function of the ionic strength of the solution.

2. It has been shown that the polyvalent anions and heparin inhibit coagulation by acting directly on thrombin. The inhibiting action of polyvalent cations was found to be due to their effect on fibrinogen.

3. An explanation of the results has been advanced on the basis that alterations of the electrical potentials of thrombin and fibrinogen interfere with the formation of a coagulation enzyme-substrate intermediate.

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THE RESPIRATION OF HUMAN SPERMATOZOA AND THEIR RESPONSE TO VARIOUS GASES AND LOW TEMPERATURES

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Iwanow reported in 1931 that dog spermatozoa continue to move for several hours under partial anaerobic conditions. This movement is unassociated with the production of carbon dioxide, because it occurs when cellular respiration is entirely inhibited by KCN. He concluded, therefore, that the energy for spermatozoan movement arises from hydrolytic phenomena and not oxidative processes. In 1935 he demonstrated that motility is retained for a long time in concentrations of monoiodo-, monobromo-, and monochloro-acetic acid sufficient to inhibit glycolysis, and therefore concluded that glycolysis cannot be an essential process for supplying the energy necessary for movement. He stated (1936) that in the presence of glucose the respiratory quotient of sheep spermatozoa is approximately 1.0 and about 0.78 in its absence, motility nevertheless being retained in the medium deprived of glucose.

Redenz (1933) in observations on bull spermatozoa concluded that under anaerobic conditions their motility is promoted by energy derived from glycolysis and under aerobic conditions the addition of sugar increases activity. He also reported that the spermatozoa of the bull utilized 9.0 cu. mm. of oxygen per milligram of dry weight per hour and those of the guinea pig 7.0. Fujita (1928) obtained the figure 7.7 for the rat.

Goldblatt (1935) found that glycolysis of human seminal fluid is demonstrable only in the presence of active spermatozoa and is associated with a large increase in lactic acid which, however, is insufficient to account for the loss of reducing power. According to him, fresh spermatozoan-free seminal fluid contains around 200 to 300 mgm. per cent of glucose. Killian (1933) showed that as a semen specimen ages, the lactic acid increases and the high sugar content decreases, particularly when more than three hours have elapsed since its collection.

Most of the semen specimens used in the experiments summarized in table 1 were furnished at intervals of about one week by three donors and were collected manually. They were tested one-half, six, and twelve hours

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after ejaculation. The count was constantly about 75 million cells per cubic centimeter for donor A, 150 for B, and 0 for C.

The rate of oxygen consumption of undiluted semen was measured at 37° ± 0.02°C. by the direct method of Warburg as described by Dixon (1934, p. 45). Measurements of respiration were made in air over a three-hour period and the semen then examined to see whether the spermatozoa were still active. The results of these experiments are presented in table 1.

In the first place, table 1 demonstrates that the spermatozoa remained active throughout the full three-hour run of the experiments. In the second place, a comparison of the oxygen consumed in the half-hour old specimens of A and B shows that the consumption is almost directly pro-

TABLE 1

Relation of the age of the semen specimen, the number of spermatozoa, and the rate of respiration

DONOR	AGE OF SPECIMEN AT BEGINNING OF EXPERIMENT	CONDITION OF SPERMATOZOA AT END OF EXPERIMENT	NUMBER OF SPERMATOZOA PER CC.	OXYGEN CONSUMED PER HALF-HOUR IN CU.MM.						AVERAGE OXYGEN CONSUMED PER HOUR OVER 3 HR. PERIOD
				30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	
	hours		*million*							*cu.mm.*
A	½	Very active	75	6.0	6.0	6.1	5.4	4.5	4.0	10.6
A	½	Very active	75	6.4	6.1	6.2	5.7	3.1	3.5	10.2
A	6	Very active	75	4.5	4.4	4.7	3.6	2.8	2.7	7.6
A	6	Very active	75	3.8	3.8	3.4	3.8	3.0	2.8	6.9
A	12	Very active	75	4.9	4.6	4.3	4.8	3.4	4.0	8.6
A	12	Very active	75	3.1	3.1	3.0	2.9	2.7	2.3	5.7
B	½	Very active	150	12.1	11.8	12.1	11.3	9.8	11.2	22.6
B	½	Very active	150	11.2	11.1	10.7	9.6	10.5	8.7	20.3
C	½		0	0	0	0	0	0	0	0
C	½		0	0	0	0	0	0	0	0

portional to the magnitude of the sperm count. In the third place, the consumption of oxygen by the spermatozoa from one of the twelve-hour old specimens was greater than that used by either of the samples six hours old. In the fourth place, there was no measurable respiration in those specimens devoid of spermatozoa. Finally, the oxygen consumption of some of the specimens decreased gradually and that of others remained fairly constant during the three-hour period of observation.

Semen specimens from donors A and D each with approximately 75 million cells per cubic centimeter were used in experiments on the respiratory quotient. They were tested one-half, six, and twelve hours after ejaculation. The respiratory quotient was measured by the first method of Dickens and Simer (Dixon, 1934, pp. 61–67). The results are given in table 2.

Table 2 shows that the respiratory quotient obtained for spermatozoa from one-half hour old specimens was 0.93 for donor A, and 0.88 and 0.87 for D. However, this individual variation in the respiratory quotient, as in the case of oxygen consumption, is not nearly so pronounced as the variation caused by ageing. The table further demonstrates that the respiratory quotient is closely correlated with the age of the specimen and varies directly with it. It also varies directly with the rate of oxygen consumption.

This direct variation of the respiratory quotient with the rate of oxygen consumption throws light on the nature of the substance oxidized during respiration by spermatozoa.

TABLE 2

Relation between the rate of consumption of oxygen by human spermatozoa and the respiratory quotient

AGE OF SEMEN SPECIMEN	DONOR	AVERAGE RATE OF CONSUMPTION OF OXYGEN OVER THREE HOUR PERIOD PER 75 MILLION SPERMATOZOA PER HOUR	RESPIRATORY QUOTIENT
hours		*cu mm.*	
$\frac{1}{2}$	A	12.7	0.93
$\frac{1}{2}$	A	12.6	0.93
$\frac{1}{2}$	D	12.3	0.88
$\frac{1}{2}$	D	12.0	0.87
6	A	8.9	0.81
6	A	7.6	0.81
6	A	9.0	0.82
12	A	6.0	0.73
12	A	5.4	0.72
12	A	5.7	0.72

If complete oxidation occurs, the ratio of carbon dioxide produced to oxygen consumed is 1.0 for carbohydrate, approximately 0.7 for fat, and 0.8 for protein. Obviously a high respiratory quotient would imply a greater oxidation of carbohydrate than if it is low. From our results, one may see that spermatozoa in a fresh specimen with a respiratory quotient of 0.93 oxidize more carbohydrate than those in an old specimen with a respiratory quotient of only 0.72.

The response of human spermatozoa to various gases. To ascertain the effects of carbon dioxide, oxygen, nitrogen, helium, nitrous oxide, alcohol vapor, and ether vapor on the spermatozoa, a hanging drop of undiluted semen with a volume of 0.02 cc. was placed on a ruled cover glass which in turn was sealed on a micro-gas chamber provided with an inlet and outlet of equal diameter. By means of a stop watch and the ruled cover glass, any change in activity could be observed and a difference in rate measured.

When subjected to atmospheres of nitrogen, nitrous oxide, helium, or air at very low pressure, the spermatozoa continued to show motility for periods which ranged between seventeen and thirty-six hours. In helium, and likewise in pure oxygen, there was marked increase in activity. For instance, the average rate of locomotion of 100 spermatozoa in undiluted semen was increased from 1.25 mm. per minute to 2.3 per minute by exposure to pure helium; similarly, exposure to oxygen increased the average rate of locomotion of 100 spermatozoa from 2 to 3.1 mm. per minute.

Samples of semen under various experimental conditions were exposed to the same pressure of pure carbon dioxide and, as can be seen in table 3, this gas invariably caused complete immobility. It was found, however, that the time required for immobilization varied with specimens from different donors, with the freshness of the specimen, with the frequency of ejaculation, and in the cells themselves within a given specimen. Only a few seconds were required to produce quiescence in some specimens whereas several minutes were necessary in others. Specimens collected once a week from three continent donors were more resistant to the gas than those collected every two weeks or twice a week from the same source. This observation suggests that there is an optimum frequency of ejaculation for the production of spermatozoa most resistant to the effects of carbon dioxide.

Extended exposure of eleven specimens to carbon dioxide was made and in seven agglutination of the spermatozoa occurred. Attempts to revive them failed. In specimens temporarily exposed to carbon dioxide, tests were made to see whether motility could be restored when the gas was replaced by nitrogen, air, or oxygen as soon as all movement ceased. Although motility is restored, probably by a washing out effect, fewer and fewer spermatozoa are restored to activity after each successive exposure to carbon dioxide and its removal by another gas.

When exposure to CO_2 was carried out in a well buffered solution, pH 7.4, reversible cessation of activity occurred just the same even though no measurable change in hydrogen ion concentration was produced. In undiluted semen, the pH shifted about one-half unit toward the acid side. These findings indicate that the toxic effect of carbon dioxide is not dependent upon its acid character; nor can anoxia be incriminated since spermatozoa, as we have noted, show motility for several hours after oxygen withdrawal. It is apparent, accordingly, that the toxic action of this gas upon spermatozoa must be ascribed to some other property, as yet unknown. These results are in keeping with those obtained by Becker (1936), who found that the toxic effect of carbon dioxide on protoplasm represented a specific lethal action coupled with an ability to penetrate cell boundaries with extreme rapidity.

Tests with ether and alcohol vapors also produced quiescence of the

TABLE 3

Response of human spermatozoa to carbon dioxide

EXPERIMENTAL CONDITION OF SPERMATOZOA	EFFECT OF CARBON DIOXIDE
5 minutes old	Complete immobility
10 minutes old	Complete immobility
2 hours old	Complete immobility
6 hours old	Complete immobility
10 hours old	Complete immobility
14 days old	Complete immobility
3,000,000 cells per cc.	Complete immobility
50,000,000 cells per cc.	Complete immobility
175,000,000 cells per cc.	Complete immobility
300,000,000 cells per cc.	Complete immobility
Spermatozoa of 2½ hours' survival in vitro	Complete immobility
Spermatozoa of 8 hours' survival in vitro	Complete immobility
Spermatozoa of 48 hours' survival in vitro	Complete immobility
4 per cent abnormal cells present	Complete immobility
20 per cent abnormal cells present	Complete immobility
Rate of locomotion of spermatozoa:	
0.5 mm. per minute	Complete immobility
3 mm. per minute	Complete immobility
Specimens from donor A	Completely immobilized in 4 seconds
Specimens from donor B	Completely immobilized in 40 seconds
Specimens from donor C	Completely immobilized in 17 seconds
Specimens from donor D	Completely immobilized in 3 minutes
Sample of semen from donor E:	
30 minutes old	Completely immobilized in 10 seconds
1 hour old	Completely immobilized in 6 seconds
3 hours old	Completely immobilized in 1.5 seconds
Sample of semen from donor F:	
Weekly	Completely immobilized in 8 seconds
Bi-weekly	Completely immobilized in 5 seconds
Semi-weekly	Completely immobilized in 4.5 seconds
Sample of semen from donor G:	
Weekly	Completely immobilized in 28 seconds
Bi-weekly	Completely immobilized in 22 seconds
Semi-weekly	Completely immobilized in 24 seconds
Sample of semen from donor H:	
Weekly	Completely immobilized in 17 seconds
Bi-weekly	Completely immobilized in 9 seconds
Semi-weekly	Completely immobilized in 11 seconds

TABLE 3—*Concluded*

EXPERIMENTAL CONDITION OF SPERMATOZOA	EFFECT OF CARBON DIOXIDE
Among various cells within a given specimen	Completely immobilized in 1–23 seconds
Extended exposure (1–9 hours) to carbon dioxide	Agglutination in 7 out of 11 cases
Carbon dioxide replaced by nitrogen, air, or oxygen soon after cessation of activity	Immediate restoration of motility
Effect of carbon dioxide on spermatozoa in a buffered solution, pH 7.4	Cessation of activity
Effect of carbon dioxide on spermatozoa in undiluted semen	Quiescence and lowering of pH by approximately ½ unit

spermatozoa, but if exposed to either for more than a few minutes they could not be revived.

Factors influencing the resistance of human spermatozoa to very low temperatures. Davenport (1897) and Jahnel (1938) found that human spermatozoa could withstand exposure to low temperatures. Luyet and Hodapp (1938) reported that frog spermatozoa could be revived after being kept in liquid air; according to them, the essential conditions favoring revival are rapid freezing and rapid thawing.

To ascertain whether human spermatozoa could be resuscitated after exposure to −79°C., different samples approximately one hour old were placed in a bath at this temperature. A column of undiluted semen was drawn up into thin-walled capillary tubes with an internal diameter of approximately 0.2 mm. After the ends were sealed, the tubes were then instantly immersed into alcohol cooled with dry ice (−79°C.). After five minutes at this temperature, the semen was thawed by placing the tubes into a water bath at 37°C. Ten minutes later the semen was examined for active spermatozoa. To observe the effect of much greater depressions of temperature, the preceding experiment was repeated with liquid nitrogen and liquid helium (−196° and −269.5°C.). In order to study the effect of prolonged exposure to low temperature, specimens of semen one hour old were kept at −79°C. for one month and in some instances for more than two months. In these tests, capillary tube preparations were thawed by placing them in a bath at 20° and 37°C., respectively. The results of these experiments are presented in table 4.

Several facts can be gleaned from this table. First, there was definite individual variation in the sensitivity of the spermatozoa to the three freezing temperatures. Second, the per cent of one-hour cells revived after five minutes of exposure was approximately constant for the three different temperatures in the same donor. Third, the injurious effects increased with the length of exposure. Fourth, the resistance of the

spermatozoa to freezing decreased with advancing the age of the specimen. We also learned that frozen semen thawed at 20°C. gives essentially the same results as at 37°C.

The spermatozoa resuscitated in these experiments appeared to be in good morphological condition, and those which regained their motility continued active for several hours. It would be of interest to know whether these cells were still capable of initiating normal development. After freezing plus drying the morphology was preserved, but there was no resuscitation.

TABLE 4

Effect of the donor, the magnitude of the temperature change, the age of the semen, and the length of exposure to a given temperature on the resistance of human spermatozoa to very low temperatures

DONOR	TIME AT −79°C.					PER CENT REVIVAL AFTER 5 MINUTES AT −196°C. FOR SPERMATOZOA 1 HOUR OLD AT BEGINNING OF EXPERIMENT	PER CENT REVIVAL AFTER 5 MINUTES AT −269.5°C. FOR SPERMATOZOA 1 HOUR OLD AT BEGINNING OF EXPERIMENT
	5 minutes			30 days	70 days		
	Age of spermatozoa at beginning of experiment (in hours)			Spermatozoa 1 hour old at beginning of experiment	Spermatozoa 1 hour old at beginning of experiment		
	1	5	10				
	Per cent revival			Per cent revival	Per cent revival		
A	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0
C	6	1	0	5	2	6	5
D	9	0	0	8	4	7	10
E	8	3	0	5	1	8	5
F	3	1	0	4	2	2	3
G	0	0	0	0	0	0	0
H	2	0	0	0	0	1	2

SUMMARY

The rate of respiration of human spermatozoa varies inversely with the age of the specimen and directly with the number of cells per unit volume. No oxygen is consumed by semen devoid of spermatozoa. The respiratory quotient of human spermatozoa varies inversely with the age of the specimen and directly with the rate of oxygen consumption, indicating a shift in the metabolites being oxidized. The age of the spermatozoa and the number of cells per unit volume may not be the only factors involved in the consumption of oxygen, for the results presented indicate that individual variation is also a factor.

Human spermatozoa remain very active for several hours in pure nitrogen, nitrous oxide, helium, and in air reduced to a very low pressure; in helium, and likewise in pure oxygen, there is marked increase in activity.

On the other hand, carbon dioxide produces complete immobility within a few minutes, the exact time varying with the donor and the freshness of the specimen, with the frequency of ejaculation, and with certain unrecognizable cell differences resident in a single specimen. The toxic effect of the gas is dependent neither upon its acid character, nor upon anoxia, but upon other properties. Motility of spermatozoa is restored when the carbon dioxide is replaced by nitrogen, air, or oxygen as soon as all movement ceases, but fewer and fewer cells are restored to activity after each additional exposure. Ether and alcohol vapors also produce quiescence, but if exposed for more than a few minutes, the spermatozoa cannot be revived.

There is marked individual variation in the sensitivity of human spermatozoa to extremely low temperatures, but the magnitude of the depression of temperature, over the range tested, does not seem to be an important factor in affecting ability for resuscitation. The less fresh the specimen, the more the spermatozoa are affected by freezing. The effect of the freezing temperature varies directly with the length of exposure. Spermatozoa which do not survive freezing are not fragmented, and those revived from very low temperatures remain active for several hours.

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